Anaerobic mixed culture processes for bio-waste valorisation (Alternative format thesis).

De Groof, Vicky

Award date:
2021

Awarding institution:
University of Bath

Anaerobic mixed culture processes for bio-waste valorisation

Vicky De Groof

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Chemical Engineering

December 2020

Declaration of any previous submission of the work

The material presented here for examination for the award of a higher degree by research has not been incorporated into a submission for another degree.

Declaration of authorship

I am the author of this thesis, and the work described therein was carried out by myself personally, with the exception of input from supervisors and colleagues which are acknowledged at the beginning of each chapter.

This thesis is dedicated to my godchild and nephew, Mathis De Groof. Now a four-year-old who through this all reminded me what actually matters in life.

Abstract

Anaerobic mixed culture processes have great potential for bio-waste valorisation, as they convert organic matter to a range of value-added compounds. Of particular interest is acidogenic fermentation that accumulates the natural intermediates of anaerobic digestion, an already established technology. Delivering product selectivity is a challenge with complex feedstock, especially when aiming for cost-effective waste management by minimising chemical addition and operational complexity. This thesis explores operating strategies to direct fermentation of food waste towards medium chain carboxylic acids (MCCA) in single-stage stirred tank reactors. MCCA are generated via microbial chain elongation and have a higher value than other fermentation products. The substrates for chain elongation, i.e., volatile fatty acids and lactic acid or ethanol, are co-generated during food waste fermentation.

A literature review on chain elongation by mixed cultures with complex organic feedstock identified a range of operating conditions and defined the research objectives of this thesis. Firstly, an operating strategy involving organic overload to drive an anaerobic digestion community to acidogenic fermentation in a semi-continuous single-stage stirred tank reactor was evaluated. Start-up at higher feed-to-microbial ratio (>5 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$) and organic loading rates (OLR) (8.5 gCOD L^{-1} d^{-1}), compared to a parallel anaerobic digester (<1 gCOD gVS^{-1}, 4.2 gCOD L^{-1} d^{-1}), inhibited methanogenesis and produced volatile fatty acids. Chain elongation was stimulated by switching to a feedstock with higher organic content to give an OLR of up to 21 ± 2 gCOD L^{-1} d^{-1} with the same hydraulic retention time (HRT, 14 days). MCCA were produced at similar concentrations to more complicated reactor systems (22 ± 4 gCOD L^{-1} for n-caproic and 7 ± 2 gCOD L^{-1} for n-caprylic acid). A specialised community formed that showed *in situ* lactic acid production followed by chain elongation. The high-COD food waste deactivated methanogenesis and biogas production in anaerobic digestion, suggesting MCCA production is a better use of this feedstock.

The OLR is determined by the organic strength of the food waste and the applied hydraulic retention time (HRT), hence the impacts of these parameters were evaluated. The main product from food waste fermentation at 12 gCOD $L^{-1}d^{-1}$ OLR and 8.5 day HRT was n-butyric acid (13 ± 2 gCOD L^{-1}). Operating at the same 8.5 day HRT but at 20 gCOD $L^{-1}d^{-1}$ OLR resulted in lactic acid accumulation (34 ± 5 gCOD L^{-1}). This is a similar OLR and lower HRT to the system that stimulated chain elongation in previous experiment. An OLR of 12 gCOD $L^{-1}d^{-1}$ and a higher HRT of 10.5 days, stimulated chain elongation (n-caproic acid up to 13.6 gCOD L^{-1}). The microbial community was determined by the operating conditions and these together determined product profiles. Longer HRT resulted in greater abundance

of lactic-acid producing genera such as *Olsenella* spp., known to aid chain elongation, and secondary fermenters such as chain elongating species. Operating at higher OLR led to greater abundance of the homolactic genus of *Lactobacillus*. Hence, the reactor operating strategy can direct product synthesis. This shows the potential for a biorefinery with a flexible product portfolio improving commercial viability and presents an opportunity to repurpose existing single-stage AD systems by adjusting operating strategy.

The literature review also suggested the potential of a semi-continuous feeding pattern to stimulate the consecutive fermentation steps required for MCCA. Hence, bi-weekly and daily feeding patterns were compared. Daily feeding resulted in a less stable process due to lactic acid accumulation, which acidified the reactor and required more pH correction. Bi-weekly feeding resulted in higher ethanol and n-caprylic acid yields. Analysis of the microbial communities and their correlation with product formation, coupled with fermentation pathway analysis, revealed a competitive interaction between homolactic *Lactobacillus* and a consortium of primary fermentation bacteria producing ethanol, acetic and lactic acid with secondary fermenters performing chain elongation. With daily feeding the homolactic *Lactobacillus* had a competitive edge. Thus, the work reveals competitive and syntrophic interactions in the mixed culture fermentation of food waste. Understanding these provides a route to optimise process design and targeted products.

An effective waste management system requires a stable outcome regardless of natural fluctuations in the feedstock. Variations of the food waste collected during the project and their impact on fermentation was assessed via batch reactor studies. Fermenting food waste as employed in anaerobic digestion recycling centres led predominantly to carboxylic acid formation. By contrast, fermentation of fresh cafeteria food waste mainly generated lactic acid. Feedstock storage and pretreatment was shown to affect fermentation and, therefore, the necessary optimal operating conditions. Batch studies also evaluated the impact of sucrose supplementation of the feedstock. Sucrose was found to destabilise fermentation and confirmed the competition between MCCA and lactic acid as the main product. Finally, immiscible, and low density oils present in some food wastes promoted partitioning and concentration of MCCA from the aqueous fermentation broth. This leads to interesting opportunities for utilising oily feedstocks and enhancing downstream processing.

The thesis concludes by proposing the necessary operating conditions to direct acidogenic fermentation towards MCCA production in a simple one-stage reactor configuration, such as existing anaerobic digestion assets. Proposals are made for advancing this research to develop a bio-waste valorisation technology that allows sustainable resource recovery contributing to a circular bio-economy.

Acknowledgements

I would like to start by thanking the fantastic four, i.e., my supervisory team. Firstly, Dr. Marta Coma, without whom I would not have ventured to Bath to commence this wonderful journey. I would like to express my sincere gratitude to Dr. Ana Lanham who so kindly adopted me into her research group. She went above and beyond by not only being an amazing supervisor but also a mentor, A-team leader, inspiration, shoulder to lean on, occasional baker, great conversationalist and joyful companion for coffees and lunches. Marta and Ana, it has been inspiring to witness your friendship and the way you both tackle whatever life throws at you. My supervisory team would not be complete without Dr. Tom Arnot and Prof. David Leak. Tom, thank you for stepping up this last year. It was a difficult year for all of us, and I truly appreciate you checking in to see how I was doing. David, thank you for your advice and guidance throughout. Many thanks to all for your supervision and the many things you taught me.

This thesis would not have been the same without the marvellous A-team (Ana's research group). Viviane and Minh, you are absolute all-stars and I will miss our group lunches. Viviane, it has been a pleasure sharing the lab, plants, great cooking and the lab radio with you. Minh, I cannot wait to read your acknowledgements and marvel at your linguistic talents. The A-team has also known other, temporary members to whom I am grateful, such as the MEng project students acknowledged for their lab work in Chapter 6.

I would like to acknowledge the technical staff at 9W and the staff at Science Stores for their assistance throughout the years. Your help has been greatly appreciated, particularly when sending biomass samples for DNA-analysis at the speed of light just before a global lockdown.

I am beyond grateful to have been given the opportunity to go on a four-month research placement at the Singapore Centre for Environmental Life Sciences Engineering (SCELSE, NTU, Singapore). A special thanks to my placement supervisor Prof. Stefan Wuertz for his invaluable advice and his welcoming research cluster. The assistance and fruitful conversations from Abeed, Sara, Nathan, Eze and many others added to this thesis and to my overall development as a researcher. In a short time, I met many wonderful people that I would like to thank for making me feel welcome, with a particular shout out to my office neighbour Azean.

I would like to acknowledge the EPSRC Centre for Doctoral Training in Sustainable and Chemical Technologies (CSCT) for not only accepting me to do an integrated PhD, but also providing various opportunities and events to grow, learn and make connections with a

whole bunch of inspiring people. Without the CSCT I would not have met my cohort (Cohort ‘16) who made moving to a new country and making friends one of the easiest things to do.

I would like to acknowledge the support and funding from Wessex Water and GENeco (Bristol, UK). In particular, the assistance and enthusiasm in our research from Tom Phelps, Wesley Wong, Dan Green and other staff and technical colleagues.

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement H2020-MSCA-CO-FUND, No 665992. This work was supported by the EPSRC under grant EP/L016354/1. Funding for the placement was received from the CSCT, the Santander Postgraduate Mobility Award and costs related to the collaborative research covered by SCELSE. Additional conference funding was granted from the Doctoral College Placement Support Fund, the CSCT and early career researchers’ funding provided by conference organisations.

I would like to thank Prof. Sarah Lebeer. Her lectures on microbiology sparked my interest in the topic. She kindly wrote a recommendation letter for me for this PhD position under a very tight deadline. Who knew it would change my life as much as it did?

I would like to thank my dear family and friends that have supported me throughout. My parents, brothers and grandparents have each shaped me in the person I am today. I have missed them dearly moving away from home and I hope I made them proud. I would like to thank all my dear friends for their continuous support. In particular a shout out to my office mates who I am pretty sure have become more than colleagues. I would also like to thank my little lockdown family at Wincham Manor, especially Georgina Mercedes Pearson Cousen. She requested to be named in full since it was her cups of tea that fuelled the writing of this thesis. However, the person who supported and took care of me the most during the final year of my PhD and to whom I am deeply grateful is Alex. Here is to moving in and writing our next chapter together.

Dissemination

Arising primarily from work presented in this thesis

Journal articles

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Medium Chain Carboxylic Acids from Complex Organic Feedstocks by Mixed Culture Fermentation," *Molecules,* vol. 24, no. 3, 398, 2019. doi:10.3390/molecules24030398.

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation," *Processes*, vol. 8, 1487, 2020. doi:10.3390/pr8111487.

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters," *Waste Management*, Submitted manuscript.

Conference contributions

1st International Chain Elongation Conference (Wageningen, The Netherlands) October 2020, Oral presentation, "Food waste served three ways: butyric, lactic or caproic acid".

8th IWA Microbial Ecology and Water Engineering Specialist Conference (Hiroshima, Japan) November 2019, Poster presentation, "Valeric and caproic acid from food waste: balancing lactic acid production and chain elongation".

Anaerobic Digestion Network Research Colloquium: Beyond Biogas (Manchester, UK) January 2019, Poster presentation, "Higher value products from AD: a review on medium chain carboxylic acids from bio-waste".

Annual Anaerobic Digestion Network Early Career Conference (York, UK) July 2018, Oral presentation, "Medium chain carboxylic acids from COD-rich food waste as sole substrate".

IWA Young Water Professionals Regional Conference BENELUX (Ghent, Belgium) July 2017, Oral presentation, "Food waste as substrate to obtain two different enriched microbiomes for model development".

Datasets

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Dataset on experimental data available in the literature on "Medium chain carboxylic acids from complex organic

feedstock by mixed culture fermentation"," ed. Bath: University of Bath Research Data Archive, 2019. doi: 10.15125/BATH-00584

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, “Dataset for "Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation"," ed. Bath: University of Bath Research Data Archive, 2020. doi: 10.15125/BATH-00896

V. De Groof, M. Coma Bech, T. Arnot, D. J. Leak, and A. Lanham, "Dataset for "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters"," ed. Bath: University of Bath Research Data Archive, 2020. doi:10.15125/BATH-00946

Raw data files of 16s rRNA amplicon sequencing have been submitted to the EMBL-EBI database under the following accession numbers:

- PRJEB3928: Anaerobic digestion and chain elongation in food waste fermentation
- PRJEB40478: Food waste acidogenic fermentation reactor microbiota

Abbreviations

(B/Y)soy	(black/yellow) soybean processing wastewater
(s)CSTR	(semi) continuous stirred tank reactor
(s/t)COD	(soluble/total) chemical oxygen demand
^{1}D	1st order hill number for effective alpha-diversity
^{2}D	2nd order hill number for effective alpha-diversity
AD	anaerobic digestion
AF	acidogenic fermentation
AFP	acidogenic fermentation potential
ASV	amplicon sequence variant
BD	anaerobic biodegradability (%)
BES	2-bromoethylsulfonate
BMP_{COD}	biochemical methane potential (mL_{CH4} $gCOD_{fed}^{-1}$)
BMP_{VS}	biochemical methane potential (mL_{CH4} gVS_{fed}^{-1})
Brew	brewery cleaning wastewater
C1	formic acid/formate
C10	n-decanoic acid/decanoate
C2	acetic acid/acetate
C3	n-propionic acid/propionate
C4	n-butyric acid/butyrate
C5	n-valeric acid/valerate
C6	n-caproic acid/caproate
C7	n-heptanoic acid/heptanoate
C8	n-caprylic acid/caprylate
C9	n-nonanoic acid/nonanoate
CA	carboxylic acid
F/M	feed-to-microbial ratio ($gCOD_{fed}$ $gVS_{inoculum}^{-1}$)
FID	flame ionization detector
FW	food waste
FW_{caf}	cafeteria food waste
FW_{dig}	food waste digestate
$FW_{post\text{-}past}$	pasteurised food waste
$FW_{pre\text{-}past}$	non-pasteurised food waste
GC	gas chromatography
HPLC	high pressure liquid chromatography
HRT	hydraulic retention time (d)
IOL	instantaneous organic load ($gCOD\ L^{-1}$)
k_h	kinetic hydrolysis constant (d^{-1})
LBR	leach bed reactor
MCCA	medium chain carboxylic acids
MMC	mixed microbial cultures
NA	not applicable
NMDS	non-metric multidimensional scaling
NP	net production rate ($gCOD\ L^{-1}d^{-1}$)
NTP	normal temperature and pressure (293.15 K, 101.325 kPA)
OFMSW	organic fraction of municipal solid waste

OLR	organic loading rate (gCOD $L^{-1}d^{-1}$)
OTU	operational taxonomic unit
PERMANOVA	permutational multivariate analysis of variance
PERMDISP	permutation analysis for homogeneity of multivariate dispersion
pH_2 / CO_2	H_2 or CO_2 partial pressure
pKa	acid dissociation constant
RI	refractor index
RO	reverse osmosis
rpm	rounds per minute
r_S	spearman's rank correlation coefficient
SBR	sequential batch reactor
SCCA	short chain carboxylic acids
sCSTR	semi-continuous stirred tank reactor
$sCSTR_{BW}$	bi-weekly fed sCSTR
$sCSTR_D$	daily fed sCSTR
S_P	selectivity of product P (%)
SRT	sludge retention time
STP	standard temperature and pressure (273.15 K, 100 kPa)
STR	stirred tank reactor
TCD	thermal conductivity detector
TS	total solids (g L^{-1} or %ww)
TSS	total suspended solids (g L^{-1} or %ww)
UR	up-flow reactor
VFA	volatile fatty acids
VS	volatile solids (g L^{-1} or %ww)
VSS	volatile suspended solids (g L^{-1} or %ww)
ww	wet weight
Y_P	net yield of product P (%)
ΔG	Gibbs free energy change

Table of Contents

List of figures

List of supplementary figures

List of tables

List of supplementary tables

Chapter 1. Introduction

This chapter outlines the research motivation and briefly introduces some key concepts for the present thesis. It also provides an overview of the thesis scope and outline.

1.1. Thesis motivation

1.1.1. Bio-waste valorisation to make the most out of food

About one third of the food produced for human consumption is lost or wasted. In Europe alone, an estimate of 88 million tonnes of food waste is produced yearly, resulting roughly from households (53%), food processing (19%), food service (12%), food production (11%) and wholesale and retail (5%) [1]. These numbers are shocking and distressing, especially when simultaneously an estimated 8.9% of the global population was undernourished in 2019 and more than 3 billion people cannot afford a healthy diet. Moreover, 21-37% of total anthropogenic greenhouse gas emissions are caused by our current linear food system [2]. It is estimated that for every 1 USD spent on food, 2 USD is incurred in societal, economic and environmental costs [3].

As a response to this unsustainable food system, organisations and policy makers worldwide have outlined various ambitions, goals and policies. These include prevention of food loss (before reaching the consumer) and waste (from consumer side), the design of healthier foods and replacing the concept of waste by one where materials and products are kept in use for as long as possible [3, 4]. For waste management this results in a hierarchy of reduction, reuse, recycling and disposal. Currently, the most predominant global waste treatment is landfilling, yet it does not result in any revenue or resource recovery [5]. Landfilling or incineration of non-separated municipal solid waste with bio-waste result in a negative impact on public health, require land availability, generate greenhouse gases, reduce potential for energy recovery and often contribute to soil and groundwater pollution [6]. Moreover, valuable resources are irrevocably withdrawn from economic and natural cycles, thus hindering the implementation of a circular bioeconomy. By the end of 2023, EU Member States are obligated to collect bio-waste separately (Directive 2018/851/EU). Thus, these waste streams will become more available for waste valorisation. The latter is defined as the industrial processing of low-value resources such as waste into products with a target market and higher value. This allows reducing consumption of raw materials, manage waste effectively and provide new business opportunities [7].

Composting and anaerobic digestion (AD) are established bio-waste valorisation technologies that result in fertilizer and biogas as products. However, these are of relatively low economic value. Instead, research is focussing on alternative technologies that obtain higher value products to improve bio-waste valorisation. The idea of relocating bio-waste from the end of the supply chain to using it as a resource for new bio-chemicals lies on the

intersection of the concepts of the Circular Economy and Bioeconomy. Technologies enabling these concepts are widely supported from global to national scale as, for instance, they align with the UN Sustainable Development Goals (e.g., SDGs 8.2, 9.4, 12.5), the EU's Circular Economy Action Plan and Bioeconomy Strategy and the UK's 25 Year Environment Plan [8-10].

1.1.2. Anaerobic mixed microbial cultures

Bio-waste, and specifically food waste, is a mixture of different and varying biodegradable organic compounds, and thus requires a flexible treatment approach. The use of anaerobic mixed microbial cultures (MMC), allows tackling complex organic feedstock, without the need for costly sterilisation or aeration. For instance, in AD the use of MMC allows the transformation of biodegradable compounds into methane, a renewable alternative to natural gas, via a cascade of fermentation steps [11]. A second example is acidogenic fermentation (AF), which is applied in processes such as dark fermentation or the carboxylate platform where biomass is biologically converted to produce hydrogen or carboxylic acids, respectively. In AF, methane generation is inhibited to induce the accumulation of other biogas such as hydrogen and liquid fermentation compounds such as volatile fatty acids (VFA), ethanol and/or lactic acid. These can be sold on the market as platform chemicals, i.e., a basic starting material for a range of chemicals and polymers and, thus, form a renewable alternative to materials from petrochemical refining [12, 13].

Chain elongation is part of the carboxylate platform and it is of particular interest to this thesis. Some bacteria have the metabolic capacity to elongate VFA in the presence of an electron donor such as lactic acid, ethanol and/or hydrogen into medium chain carboxylic acids (MCCA) [14]. The first reports on production of MCCA by MMC date back as far as the mid-19th Century and were spontaneous observations made from curiosity-driven research [15]. During the past decades, the topic rapidly gained interest again from modern research driven by the urge to develop new ways for bio-waste valorisation. For instance, the MixAlco process developed at Texas A&M University converts bio-wastes into mixed alcohol fuels via the intermediate production of a mixture of carboxylic acids by MMC [16]. The interest for MCCA specifically, was spearheaded by a finding from a Ph.D. student at the University of Wageningen who during their work on MMC fermentation from bio-waste found that MCCA were produced from elongation of acetic acid with ethanol and characterised the bacteria responsible for it [17, 18]. This re-found research interest in chain elongation has since led to several university spin-offs, the first semi-commercial pilot plants and the early integration of this technology in bio-refineries, e.g., Capro-X, Chaincraft and Urbiofin [19-21].

1.2. Problem statement

Acidogenic fermentation provides more economically attractive products compared to traditional bio-waste recycling technologies such as heat from incineration or methane from AD. Thus, it could incentivise recycling and contribute to the development of a circular bioeconomy. However, several challenges hinder its scale-up into a viable commercial technology including effective reactor design and choice of feedstock, optimal operating parameters to direct MMC towards maximal yields of desired products and more cost-effective downstream processing [22].

MCCA in particular, compared to other products of acidogenic fermentation, present a lower solubility in water, especially when acidified, which provides opportunities for product recovery [23, 24]. Furthermore, MCCA are more reduced, thus more suitable as replacement for fossil fuel based chemicals [25]. Current chain elongation processes under development either rely on a well-defined bio-waste feedstock enriched in single substrates (e.g., whey) and/or the addition of chemicals in multi-step systems to ensure a selective product. How to obtain MCCA from complex bio-waste with mixed substrates, such as food waste, while minimising chemical addition or operational complexity, as would be required to obtain a cost-effective waste management system that could easily be adopted by industry, remains largely unknown.

1.3. Thesis scope and general objectives

This thesis was performed in partnership with Wessex Water and GENeco (Avonmouth, UK), the latter being a sewage sludge and food waste AD recycling facility. Our collaboration placed the focus on products from AF since they are natural intermediates in AD, thus presenting an opportunity to repurpose existing AD assets and diversify the product range for waste valorisation. Food waste was our target real complex feedstock because of its availability via the partnership and its high potential for carboxylate production. A simple operational setup was selected for experiments, namely stirred tank reactors (STR), as it resembles current AD systems at GENeco and thus, if successful, provides better chances to replicate operation at full-scale. Special attention was given to chain elongation products being a relevant niche in current valorisation research and they are potentially easier to separate and have a higher market value than other AF products. Therefore, the aim became to evaluate the production of MCCA from mixed culture food waste fermentation.

This was further divided into three general goals:

1. Acquire knowledge regarding chain elongation in mixed culture fermentation of complex organic feedstock.
2. Identify operating strategies that enable chain elongation of food waste while minimising chemical addition in a simple STR.
3. Investigate the underlying fermentation pathways and MMC composition in the fermentation of food waste related to MCCA production.

The second chapter of this thesis mainly tackles the first goal by providing a comprehensive literature study on MCCA production in MMC fermentation of complex organic feedstock. This literature review allowed defining more specific research objectives, pertaining to each of these three goals, presented at the end of that chapter. These objectives were then addressed in the following four research chapters. The final chapter contains a concluding summary of the main findings and how they influence future work.

References

[1] Å. Stenmarck, C. Jensen, T. Quested, and G. Moates, "Estimates of European food waste levels," Fusions EU, Stockholm978-91-88319-01-2, 2016.

[2] FAO, IFAD, UNICEF, WFP, and WHO, *The State of Food Security and Nutrition in the World 2020. Transforming food systems for affordable healthy diets*. Rome, FAO, 2020.

[3] Ellen Macarthur Foundation, "Cities and circular economy for food," 2019.

[4] D. A. Teigiserova, L. Hamelin, and M. Thomsen, "Review of high-value food waste and food residues biorefineries with focus on unavoidable wastes from processing," *Resources, Conservation and Recycling,* vol. 149, pp. 413-426, 2019.

[5] D. Hoornweg and P. Bhada-Tata, "What a waste: A global review of solid waste management," in "Urban Development Series," The World Bank2012.

[6] A. Kumar and S. R. Samadder, "A review on technological options of waste to energy for effective management of municipal solid waste," *Waste Manag,* vol. 69, pp. 407-422, Nov 2017.

[7] J. D. Kabongo, "Waste Valorization," in *Encyclopedia of Corporate Social Responsibility*, S. O. Idowu, N. Capaldi, L. Zu, and A. Das Gupta, Eds., ed: Springer, Berlin, Heidelberg, 2013, pp. 2701-2706.

[8] United Nations. (30/07/3030). *About the Sustainable Development Goals*. Available: https://www.un.org/sustainabledevelopment/sustainable-development-goals/

[9] European Commission, "A sustainable bioeconomy for Europe: strengthening the connection between economy, society and the environment," in "Updated Bioeconomy Strategy," Brussels, Belgium2018.

[10] HM Government and F. R. A. Department for Environment, "A Green Future: Our 25 Year Plan to Improve the Environment," Defra, London2018.

[11] D. J. Batstone *et al.*, "The IWA Anaerobic Digestion Model No 1 (ADM1)," *Water Science and Technology,* vol. 45, no. 10, pp. 65-73, 2002.

[12] M. T. Agler, B. A. Wrenn, S. H. Zinder, and L. T. Angenent, "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform," *Trends Biotechnol,* vol. 29, no. 2, pp. 70-8, Feb 2011.
[13] J.-R. Bastidas-Oyanedel, F. Bonk, M. H. Thomsen, and J. E. Schmidt, "Dark fermentation biorefinery in the present and future (bio)chemical industry," *Reviews in Environmental Science and Bio/Technology,* vol. 14, no. 3, pp. 473-498, 2015.
[14] C. M. Spirito, H. Richter, K. Rabaey, A. J. Stams, and L. T. Angenent, "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr Opin Biotechnol,* vol. 27, pp. 115-22, Jun 2014.
[15] A. Bechamp, "Lettre de M.A. Bechamp a M. Dumas," vol. 6, ed. Paris, France: Annales de chimie et de physique, Gallica Bibliothèque de Météo-France, 1867.
[16] M. T. Holtzapple *et al.*, "Biomass Conversion to Mixed Alcohol Fuels using the MixAlco process," *Applied Biochemistry and Biotechnology,* vol. 77-79, pp. 609-631, 1999.
[17] Wageningen University & Research. (11/11/2020). *3 ETE discoveries of the past 50 years: 3. From organic waste to high-quality chemicals*. Available: https://www.wur.nl/en/article/3-ETE-discoveries-of-the-past-50-years-3.-From-organic-waste-to-high-quality-chemicals.htm
[18] K. J. J. Steinbusch, H. V. M. Hamelers, C. M. Plugge, and C. J. N. Buisman, "Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass," *Energy Environ. Sci.,* vol. 4, no. 1, pp. 216-224, 2011.
[19] Chaincraft. (30/07/2020). *ChainCraft: What if waste can become a new product?* Available: https://www.chaincraft.nl/
[20] Urbiofin. (30/07/2020). *Urban Biorefinery: From waste to bioproducts through biorefinery*. Available: https://www.urbiofin.eu/
[21] Capro-X Inc. (2020, 11/11/2020). *Capro-X the sustainable way for whey*. Available: http://www.capro-x.com/
[22] A. H. Bhatt, Z. J. Ren, and L. Tao, "Value Proposition of Untapped Wet Wastes: Carboxylic Acid Production through Anaerobic Digestion," *iScience,* vol. 23, no. 6, p. 101221, Jun 26 2020.
[23] S. Gildemyn, B. Molitor, J. G. Usack, M. Nguyen, K. Rabaey, and L. T. Angenent, "Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation," *Biotechnol Biofuels,* vol. 10, p. 83, 2017.
[24] J. Xu, J. J. Guzman, S. J. Andersen, K. Rabaey, and L. T. Angenent, "In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis," *Chem Commun (Camb),* vol. 51, no. 31, pp. 6847-50, Apr 21 2015.
[25] S. Dahiya, A. N. Kumar, J. Shanthi Sravan, S. Chatterjee, O. Sarkar, and S. V. Mohan, "Food waste biorefinery: Sustainable strategy for circular bioeconomy," *Bioresour Technol,* vol. 248, no. Pt A, pp. 2-12, Jan 2018.

Chapter 2. Literature review: Medium chain carboxylic acids from complex organic feedstock by mixed culture fermentation

The following chapter provides a systematic analysis of the literature to address the first general goal of this thesis, knowledge acquisition regarding chain elongation in mixed culture fermentation of a complex organic feedstock. A dataset was prepared to evaluate the literature that reported medium chain carboxylic acids as either main- or by-product. This dataset formed the backbone of the literature discussion and the generated figures. It resulted in a comprehensive overview on chain elongation in microbial culture fermentation and the various tried operational parameters to produce medium chain carboxylic acids as added-value product from a complex organic feedstock.

A section has been included with an update of the literature that was published between the publication of this chapter as a review paper and the submission of the present thesis manuscript.

The conclusion from this chapter allowed mapping out specific research objectives, which are presented at the end of this chapter.

This chapter is submitted in an alternative thesis format in line with Appendix 6A of the "Specifications for Higher Degree Theses and Portfolios" as required by the University of Bath. This literature review was published in MDPI Molecules' Special Issue "Chemicals from Food Supply Chain By-Products and Waste Streams":

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Medium Chain Carboxylic Acids from Complex Organic Feedstocks by Mixed Culture Fermentation," *Molecules,* vol. 24, no. 3, 398, 2019. doi.org:10.3390/molecules24030398.

The dataset was made available as:

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Dataset on experimental data available in the literature on "Medium chain carboxylic acids from complex organic feedstock by mixed culture fermentation"". *University of Bath*, 22 Jan 2019. doi: 10.15125/BATH-00584

Author Contributions: conceptualization, V.D.G., M.C. and A.B.L.; data curation, V.D.G.; writing—original draft preparation, V.D.G.; writing—review and editing, M.C., D.J.L., A.B.L., T.C.A.; supervision, A.B.L.; project administration, A.B.L.; funding acquisition, T.C.

This declaration concerns the article entitled:			
Medium chain carboxylic acids from complex organic feedstock by mixed culture fermentation			
Publication status (tick one)			
~~**Draft manuscript**~~	~~**Submitted**~~	~~**In review**~~	~~**Accepted**~~ **Published**
Publication details (reference)	V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Medium Chain Carboxylic Acids from Complex Organic Feedstocks by Mixed Culture Fermentation," Molecules, vol. 24, no. 3, 398, 2019. doi:10.3390/molecules24030398.		
Copyright status (tick the appropriate statement)			
I hold the copyright for this material	~~Copyright is retained by the publisher, but I have been given permission to replicate the material here~~		
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: The candidate predominantly formulated the ideas. These were further developed with input from and in discussion with co-authors. – 70% Design of methodology: The candidate predominantly designed the methodology. Methodology was optimized and developed further in discussion with co-authors – 80% Experimental work: The candidate predominantly executed data curation – 100% Presentation of data in journal format: The candidate wrote the original draft of the paper. Co-authors reviewed and proposed edits. – 70%		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	Vicky De Groof	**Date**	26th March 2021

2.1. Published literature review: Medium chain carboxylic acids from complex organic feedstock by mixed culture fermentation

Vicky De Groof[1,4], Marta Coma[1], Tom Arnot[2,4], David Leak[3], Ana Lanham [2,4],*

[1] EPSRC Centre for Doctoral Training in Sustainable Chemical Technologies, University of Bath, Claverton Down, Bath, BA2 7AY, UK

[2] Water Innovation & Research Centre, University of Bath, Claverton Down, Bath, BA2 7AY, UK

[3] Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK

[4] Department of Chemical Engineering, University of Bath, Claverton Down, Bath, BA2 7AY, Bath, UK

* Correspondence: a.lanham@bath.ac.uk Tel.: +44-1225- 384544

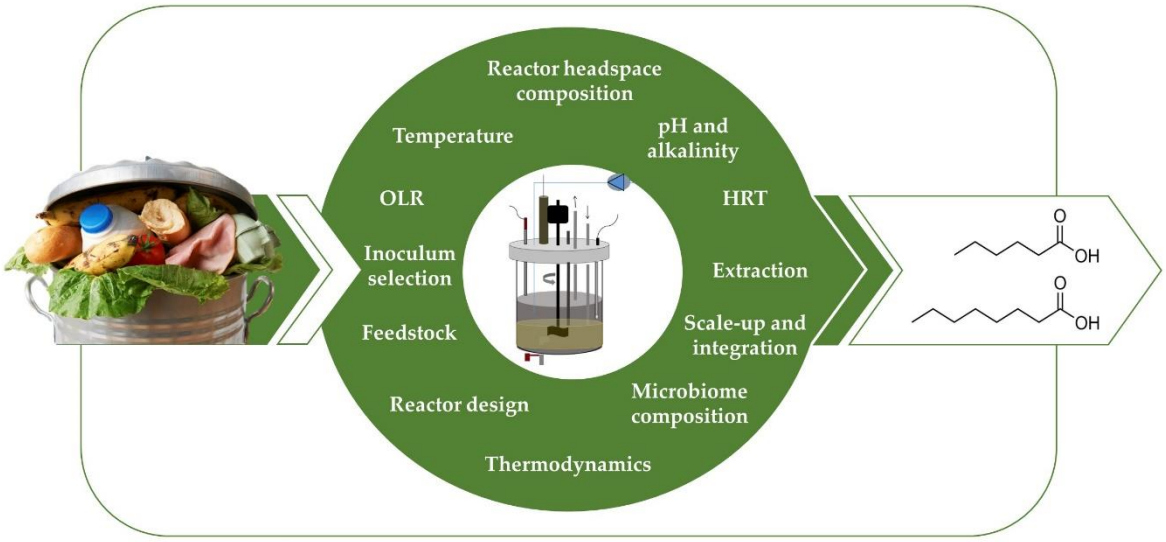
Academic editor: Afroditi Chatzifragkou

Received: 30 November 2018; Accepted: 18 January 2019; Published: 22 January 2019

Abstract

Environmental pressures caused by population growth and consumerism require the development of resource recovery from waste, hence a circular economy approach. The production of chemicals and fuels from organic waste using mixed microbial cultures (MMC) has become promising. MMC use the synergy of bio-catalytic activities from different microorganisms to transform complex organic feedstock, such as by-products from food production and food waste. In the absence of oxygen, the feedstock can be converted into biogas through the established anaerobic digestion (AD) approach. The potential of MMC has shifted to production of intermediate AD compounds as precursors for renewable chemicals. A particular set of anaerobic pathways in MMC fermentation, known as chain elongation, can occur under specific conditions producing medium chain carboxylic acids (MCCA) with higher value than biogas and broader applicability. This review introduces the chain elongation pathway and other bio-reactions occurring during MMC fermentation. We present an overview of the complex feedstocks used, and pinpoint the main operational parameters for MCCA production such as temperature, pH, loading rates, inoculum, headspace composition, and reactor design. The review evaluates the key findings of MCCA production using MMC, and concludes by identifying critical research targets to drive forward this promising technology as a valorisation method for complex organic waste.

Graphical abstract



Keywords

Anaerobic

Carboxylate platform

Chain elongation

Circular economy

Mixed microbial culture

Medium chain carboxylic acid

Organic waste

Resource recovery

Waste valorisation

2.1.1. Introduction

In 2016 nearly 58% of the organic fraction of municipal solid waste (OFMSW) in the EU was sent directly to landfill or incineration[1], resulting in undesirable environmental effects, little to no value recovery, and hence a loss of resources. However, recycling in the EU is now increasing, and hence separately collected organic waste is becoming more available for resource recovery or waste valorisation, i.e., the process of converting waste into energy, chemicals or materials [1]. Technologies for bio-waste valorisation can be categorised as thermal or thermochemical such as hydrothermal liquefaction, pyrolysis and gasification, physicochemical like extraction and transesterification or biological conversion processes [2, 3]. Biomass gasification has been proposed to homogenise various substrates to syngas and further process this for chemical production [4]. Reviews are available regarding technologies for waste to energy [5, 6], or waste to chemicals and materials [7-10]. The choice of treatment method will depend on several factors such as type and availability of organic waste streams, e.g., the waste's organic strength measured by chemical oxygen demand (COD) [11], relative content of biopolymers (i.e., cellulose, hemicellulose or lignin) [12], or biomass type (woody biomass, types of agricultural residues, household organic waste and sewage sludge)[13, 14]. Development of a circular economy where waste is used as resource for renewable energy and chemicals will require the integration of different types of conversion processes to deal with the complexity of bio-waste and maximize resource recovery [15].

Established bio-waste valorisation technologies are composting and anaerobic digestion (AD), which each produce fertilizer and methane-rich biogas as end-products. However, the final products have relatively low economic value. For instance only €2 worth of compost is obtained per tonne food waste [16]. AD generates a slightly more valuable product: assuming the OFMSW typically contains 306.4 gCOD kg^{-1} of anaerobic biodegradable content [17] and that biogas conversion yields €0.25 worth of biogas per kg of COD [18], then a tonne of food waste will produce about €76 worth of biogas. However, the intermediate fermentation compounds produced during AD have a higher market value.

Fermentation to accumulate the intermediate carboxylates is known as the carboxylate platform. Producing carboxylates through fermentation forms a sustainable alternative to their current production from fossil fuels or extraction in small amounts from natural oils [19].

[1]Estimated using Eurostat database accessed 28/11/2018: Recycling of bio-waste (cei_wm030), Generation of waste by waste category (ten00108) and Population on 1 January (tps00001).

Compared to AD, the carboxylate platform shows lower conversion yields, yet the higher product value and broader applications can result in a higher economic value [20]. In the last decade, particular interest has grown in medium chain carboxylic acids (MCCA). They are defined as carboxylic acids with an aliphatic straight carbon chain of 6 to 12 carbon atoms, e.g., n-caproic acid has a straight chain of 6 carbon atoms (C6). MCCA are more hydrophobic compared to shorter chain carboxylates, which makes them a more interesting fermentation product as it facilitates recovery from the fermentation broth [21]. In terms of potential value, C6 has a market size of 25,000 tonne per year, with an unrefined value of $1,000, and refined value of $2,000 to $3,000 per tonne [22, 23]. Overall, MCCA have a wide range of applications: they can be applied as growth-promoting antibiotic replacements in animal feed [24, 25], or be converted via various bio-, thermo-, or electro-chemical processes into bulk fuels or solvents [14, 26-28]. The production of MCCA as higher value products from organic waste can incentivise for improved recycling while simultaneously replacing current unsustainable production processes.

MCCA are produced by certain bacteria in a strongly reduced anaerobic environment, via a metabolic pathway that has been recently reviewed by Spirito et al. [29]. The bacteria gain energy by combining the oxidation of an electron donor, i.e., lactic acid or ethanol, to acetyl-CoA with the reductive elongation of acetyl-CoA with acetic acid (C2), propionic acid (C3), butyric acid (C4), pentanoic acid (C5), or caproic acid (C6) generating a carboxylic acid with 2 additional carbons at each step (Figure 2-1). The reduction step is required to provide sufficient Gibbs free energy (ΔG) to generate ATP in the initial oxidation step, restore the NAD^+/NADH balance in the cell, and contribute to further energy generation via electron-transport phosphorylation. Ethanol and lactic acid have similar thermodynamic capacity to act as electron donors [30]. This chain elongation pathway is called the reverse β-oxidation pathway, since it is seen as the reversed biochemical degradation or β-oxidation of fatty acids.

Instead of using pure or engineered cultures, a consortium of microorganisms has more potential to deal with complex and variable feedstock such as organic waste. Mixed microbial cultures (MMC), also referred to as microbiomes, are communities of microorganisms within a well-defined environment of specific physicochemical properties [31]. Microbiomes are employed in biotechnology, for example in anaerobic digestion (AD), and in bioremediation by cultivating communities within contaminated soils [32, 33]. The term “microbiome” is used to describe the mixed microbial communities related to the human and animal gut, mouth or skin, or plant rhizospheres.

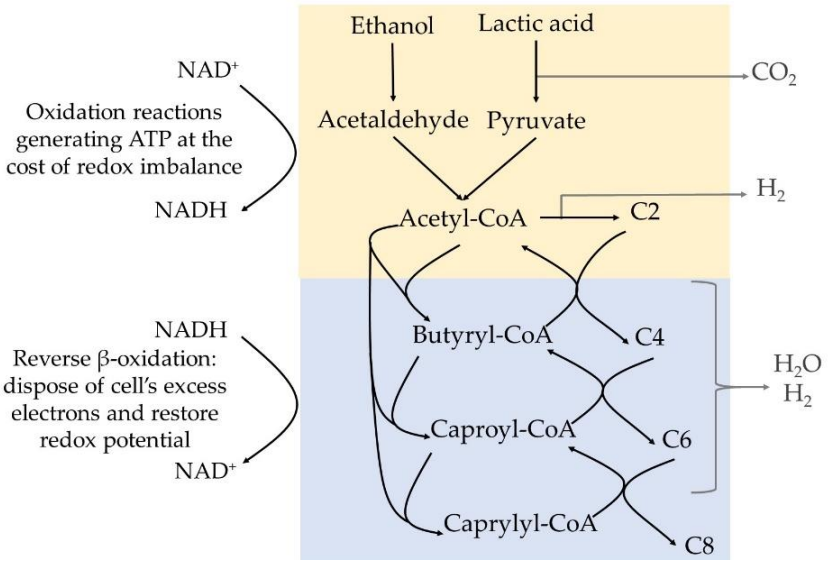


Figure 2-1 *Simplified chain elongation pathways using ethanol or lactic acid as electron donors based on the metabolic pathways described in [29].*

The first report of MMC that produced MCCA dates back to the mid-19th Century, where Béchamp attributed the production of approx. 6 gCOD L^{-1} C6 from ethanol, meat extract and chalk in a fermentation reactor to microbial activity [34]. A few decades later in the early 20th Century, an oily, immiscible layer comprising 5.3 gCOD L^{-1} C4 and 6.4 gCOD L^{-1} C6 was produced in a 30-day fermentation with impure cultures from a nutrient medium containing 24 gCOD L^{-1} ethanol [35]. Further microscopic study of the fermentation sludge revealed a consortia of microorganisms comprising methanogenic archaea and spore-forming bacteria [35]. By contrast with pure cultures, MMC do not require sterilisation, can degrade a complex feedstock, show a resilience to operational upsets [36], and allow continuous, long-term operation [37]. These advantages provide a strong argument for utilising microbiomes.

In MMC, conversion of organic substrates occurs following a cascade of steps catalysed by different microorganisms that form synergistic and competitive interactions, resulting in a complex microbial ecosystem with a versatile metabolic capacity [38]. The different microbial groups can convert organic molecules into substrates available for chain elongating bacteria. In general, biodegradable organics are hydrolysed and fermented to intermediate compounds that acidify the medium, i.e., acidogenesis, including hydrogen gas (H_2), lactic acid, ethanol, formic acid (C1) and volatile fatty acids (VFA), i.e., straight short chain carboxylic acids with 2 to 4 carbon atoms. The accumulated intermediates can undergo several secondary bioconversion steps, including chain elongation to produce MCCA (Figure 2-2) [26]. For instance, co-culture of the chain elongating bacteria *Clostridium kluyveri* with specific cellulolytic species or a rumen microbiome showed chain elongation potential from a cellulose substrate and ethanol [39, 40]. The supporting

community can even be designed or selected to allow chain elongation from a specific compound, such as glycerol or syngas (CO) [41-43], or allow the use of alternative electron donors such as, for instance, the cathode in a bio-electrochemical system [44, 45].

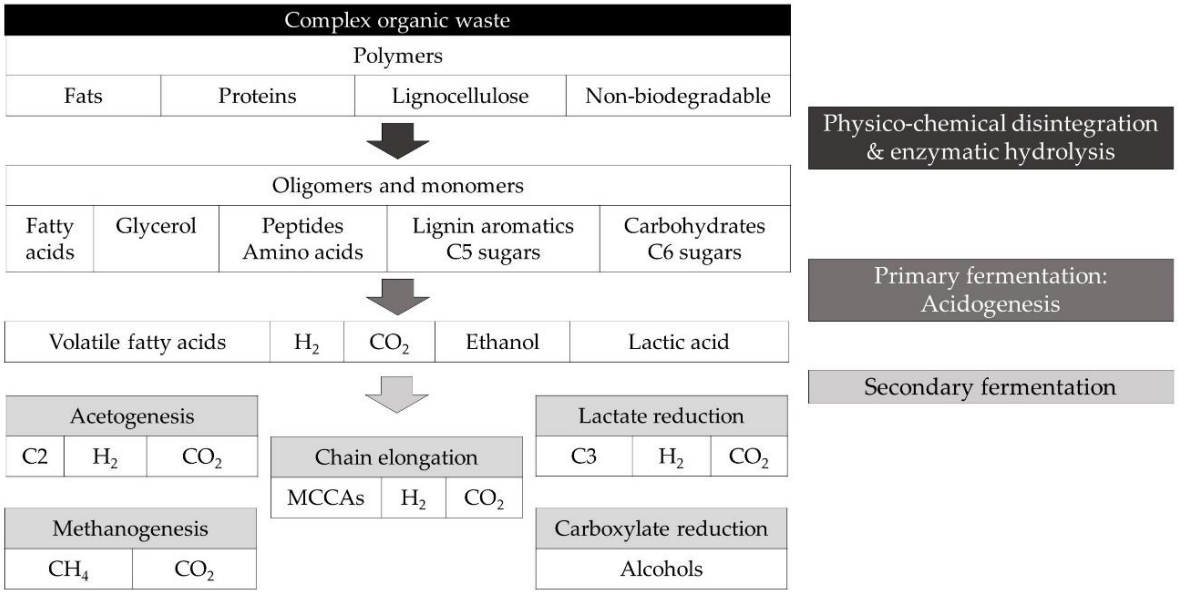


Figure 2-2 *Simplified overview of fermentation pathways that can occur in MMC.*

While it is generally believed that specific operational conditions allow development of a MMC for a functional and stable process [46], the broad metabolic capacity also gives rise to a set of various competitive reactions and by-products, especially when utilising a complex feedstock. Manipulating the environmental conditions, by regulating operation, allows some control to be exerted on the product spectrum, as it affects the thermodynamics of conversion processes, and therefore the microbiome composition that catalyses these conversions. However, current knowledge of control over the product outcome to improve MCCA yields in MMC fermentation is limited since experiments that use complex feedstock for MCCA production have only emerged in the past few years.

While the operational conditions that select for other MMC fermentation products such as VFA [47] and H_2 [48] have been reviewed, the operational conditions or process set-up that allow MCC to be steered towards MCCA formation have to be further evaluated. A recent review is available regarding the use of bio-electrochemical systems for MCCA production as a complementary technology to AD [49]. Certain other reviews include a section on MCCA as potential MMC fermentation products, either in the context of operational control applied in AD [50], or the contexts of a bio-refinery [51], wastewater treatment [11] or food waste treatment [21, 52-54]. However, a focussed analysis of the literature to identify and connect key operational parameters to target MCCA production from MMC fermentation of complex feedstocks is lacking. Therefore, this work aims to analyse the current literature,

and hence complement existing reviews. For this, studies were included that specifically target chain elongation, but the scope was extended to include other MMC-based studies that have noted MCCA as by-products from, for instance, VFA or H_2 production. Concentrations and production rates are converted to a COD-basis to allow comparison between studies using different reporting concentrations (supplementary information Table S2-1). The review evaluates the key operational parameters for MCCA production from complex substrates using MMC, with the objective of stimulating and accelerating research to produce sustainable, bio-based fuels and chemicals from organic waste. In addition, a database was generated from the experimental data available in the literature regarding MCCA production using MMC fermentation [55].

2.1.2. Chain elongation behaviour of pure cultures can be extended for MMC

Chain elongation via ethanol is the most studied pathway to date. The mechanism has been elucidated by studying *Clostridium kluyveri*, a gram-positive, spore-forming bacteria from the phylum of Firmicutes whose whole genome has been published [56]. For each molecule of ethanol oxidized to C2, resulting in substrate-level ATP-generation and production of H_2, 5 molecules of ethanol enter the reverse β-oxidation pathway as acetyl-CoA and elongate 5 molecules of C2 to C4. Subsequently, C4 can be elongated to C6 via ethanol-derived acetyl-CoA addition (Table 2-1, Eq. 1 and 2) [29]. In reality, the pathway of *C. kluyveri* has a more flexible stoichiometry influenced by substrate concentrations, ratio of ethanol to acetate, and the partial pressure of H_2 (Table 2-1, Eq. 3 to 6)[57-59]. It also has a broader substrate range including propanol as an electron donor, or propionate (C3), succinate, malonate, 3-butenoate, 4-hydroxybutyrate and crotonate as electron acceptors [39, 60, 61]. Pure culture fermentations of *C. kluyveri* fed with ethanol and C2 mixtures have been reported to produce C6 up to 10.2 gCOD $L^{-1}d^{-1}$ in continuous culture [62] and to reach concentrations up to 30.7 gCOD L^{-1} after 72 h of batch culture [60].

Chain elongation via lactic acid has been reported for other bacteria in the phylum of Firmicutes, such as *Megasphaera elsdenii* [63] and a *Ruminococcaceae* bacterium CPB6 [64]. Other wild-type bacteria are known to perform chain elongation and produce C6 (and C8) using more “exotic” chain elongating substrates such as simple sugars, polyols, methanol, amino acids and H_2 and CO_2 gas mixtures as reviewed by Angenent et al. [58]. In addition, pathways have been engineered to produce C6. For instance, to improve yields the genes from *Megasphaera* sp. were expressed in *Escherichia coli*, and approx. 1.17 gCOD $L^{-1}d^{-1}$ C6 was obtained after 36 h of batch fermentation [65]. To develop a more

thermo-tolerant and acid-resistant biocatalyst, biosynthetic pathways have been constructed in the yeast *Kluyveromyces marxianus* [66]. Single-strains or engineered cultures have their place when the product is of high enough value and requires a certain purity. Overall, the production of MCCA, and other medium chain chemicals, using pure, engineered cultures has been recently reviewed by other authors, e.g., Sarria et al. [67] and Su et al. [68].

Table 2-1 Chain elongation reactions via ethanol and lactic acid and thermodynamic information with concentrations and pressures of all components at 1 M or 1 bar, pH 7 at 25 °C.

Equation	Chain Elongation Stoichiometry	$\Delta G_r°$ [kJ mol^{-1}]	Ref
	Via Ethanol - Coupled Reactions: As Determined in *C. kluyveri*		
	Overall chain elongation to C4		
1.a	1× - Ethanol oxidation ($CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2$) ×1	10.50	[29]
1.b	5× - Reverse β-oxidation to C4 ($CH_3CH_2OH + CH_3COO^- \rightarrow CH_3(CH_2)_2COO^- + H_2O$) ×5	-193.00	[29]
1.	→ $6\ CH_3CH_2OH + 4\ CH_3COO^- \rightarrow 5\ CH_3(CH_2)_2COO^- + H^+ + 2\ H_2 + 4H_2O$	-182.50	[29]
	Overall chain elongation to C6		
2.a	1× - Ethanol oxidation ($CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2$) ×1	10.50	[29]
2.b	5× - Reverse β-oxidation to C6 ($CH_3CH_2OH + CH_3COO^- \rightarrow CH_3(CH_2)_4COO^- + H_2O$) ×5	-194.00	[29]
2.	→ $6\ CH_3CH_2OH + 5\ CH_3(CH_2)_4COO^- \rightarrow CH_3COO^- + 5\ CH_3(CH_2)_4COO^- + H^+ + 2\ H_2 + 4\ H_2O$	-183.50	[29]
	Via Ethanol - Alternative Stoichiometry		
	Overall chain elongation to C6		
3.	$12\ CH_3CH_2OH + 3\ CH_3COO^- \rightarrow 5\ CH_3(CH_2)_4COO^- + 4\ H_2 + 8\ H_2O + 2\ H^+$	-30.55	[69]
4.	$6\ CH_3CH_2OH + 3\ CH_3COO^- \rightarrow CH_3(CH_2)_2COO^- + CH_3(CH_2)_4COO^- + 2\ H_2 + 4\ H_2O + H^+$	-183.00	[56]
5.	$CH_3COO^- + 2\ CH_3CH_2OH \rightarrow CH_3(CH_2)_4COO^- + 2\ H_2O$	-79.00	[70]
	Reverse β-oxidation to C8		
6.	$CH_3CH_2OH + CH_3(CH_2)_4COO^- \rightarrow CH_3(CH_2)_6COO^- + H_2O$	NS	[62]
	Via lactic acid		
7.	Lactic acid to C2 for ATP generation $CH_3CH_2(OH)COO^- + H_2O \rightarrow CH_3COO^- + 2\ H_2 + CO_2$	-8.79	[69]
8.	Overall chain elongation to C4 $CH_3CH_2(OH)COO^- + CH_3COO^- + H^+ \rightarrow CH_3(CH_2)_2COO^- + H_2O + CO_2$	-57.52	[69]
9.	Overall chain elongation to C6: as determined for *M. elsdenii* $CH_3CH_2(OH)COO^- + CH_3(CH_2)_2COO^- + H^+ \rightarrow CH_3(CH_2)_4COO^- + H_2O + CO_2$	-57.65	[69]

NS, not specified

However, when it comes to breaking down a complex feedstock such as organic waste, the focus of this review, pure cultures have limited metabolic capacity, reducing their potential for an effective treatment and requiring more expensive processing such as media sterilisation [71]. This can be circumvented by using MMC instead of pure cultures. Chain elongation in MMC happens in a similar manner than with pure cultures. For instance, microbiomes grown in ethanol-rich conditions show similar characteristics to pure culture fermentation, such as higher specificity towards longer chain carboxylates at higher ethanol/acetate ratios [72, 73] and elongation towards a mixture of even- and uneven MCCA in the presence of propanol or C3 [74, 75]. It should be noted that MMC are unable to use either 4-carbon alcohols or 5-carbon carboxylates as initial substrate sources for chain elongation at similar concentrations than for example ethanol or acetate [75]. This may be due to longer chain substrates having higher toxicity and possible inhibition of the microbiome. In addition, microbiomes are capable of adapting to substrate fluctuations: MMC obtained from ethanol-based chain elongation reactors acclimatised to produce C6 when fed with methanol or lactic acid as an alternate electron donor [76, 77].

2.1.3. Thermodynamic models and MMC composition determine competitive processes

Successful production of MCCA requires elimination of competing reactions that could consume the substrate or product. Some example reactions include methanogenesis, sulphate reduction, lactate reduction to propionate (C3), excessive oxidation of ethanol and reduction or oxidation of carboxylic acids, and are described in Table 2-2. Since anaerobic ecosystems are energy-limited with ΔG of conversion processes being close to 0 kJ mol^{-1} (Table 2-1 and Table 2-2) [78], the thermodynamic favourability of bioconversion processes can shift by small changes in substrate or product concentrations, pH and temperature, partial pressure of gases in reactor headspace, or substrate availability [50, 79, 80]. The resulting thermodynamic constraints select for the viable bioconversion reactions and, hence, the composition of microbiome that has the most efficient catabolic system [81, 82]. Therefore, strategies to inhibit competitive reactions can be classified as; (i) the inhibition of a specific, competitive trophic group, or (ii) the engineering of the fermentation environment to reduce the potential competitive reactions. For instance, methanogenesis, the ability to produce CH_4, is limited to certain archaea. Since CH_4 has the lowest free energy content per electron upon oxidation to CO_2 under anaerobic conditions, and automatically leaves the reactor as a gas, it will be produced by methanogens in MMC to optimally use the energy available [71]. To ensure that C2 or H_2 are not lost to CH_4 and CO_2 (Table 2-2, Eq. 10), specific methanogenic inhibitors can be added to promote for chain

elongation. For instance, in batch fermentation of a synthetic substrate containing ethanol and C2, the addition of 2-bromoethylsulfonate (BES) tripled C6 production to 19 gCOD L^{-1} [83]. Alternatively, to avoid the cost of such chemicals, specific operational conditions such as pH or hydraulic retention time (HRT) can be selected to inhibit methanogens as discussed subsequently. Another unwanted trophic group are the sulphate reducing bacteria. A sulphur-rich feedstock will result in sulphate reduction, as this is more thermodynamically favourable than C6 production (Table 2-2, Eq. 11), generating sulphide, which is both toxic for most bacteria and corrosive to fermentation equipment [84].

Thermodynamic models are useful tools to improve understanding of the chain elongation pathway in MMC and to determine which operational parameters allow to regulate the product spectrum. Research has developed kinetic and thermodynamic models based on pure culture chain elongation using *C. kluyveri* [69, 85] or metabolic energy-based models to predict MMC fermentation of simple substrates such as glucose [86]. Such models can help understanding the occurrence of chain elongation at different ethanol concentrations [58], or at varying H_2:CO_2 ratios [30]. There is a lack of models that evaluate the thermodynamics of the lactic acid-based chain elongation route. At standard conditions lactate reduction (Table 2-2, Eq. 12) releases more energy than chain elongation via lactic acid (Table 2-1, Eq. 9). Experimentally, Kucek et al. [77] found increasing lactate loading rate with a synthetic feedstock initially improved chain elongation in MMC fermentation, yet increasing influent lactic acid from 9.1 to 16.2 gCOD $L^{-1}d^{-1}$ led to a collapse of C6 productivity to 3.0 gCOD $L^{-1}d^{-1}$ while C3 production increased to 5.5 gCOD $L^{-1}d^{-1}$. This was attributed to the competitive acrylate pathway being stimulated ahead of chain elongation at elevated lactic acid concentrations [77, 87]. In contrast, another study operating with an excess of lactic acid did not report C3 production; the addition of three spikes in a fed batch-style adding a total about 26.7 gCOD L^{-1} lactic acid to the synthetic medium resulted in C6 accumulation of up to 51.7 gCOD L^{-1} [88]. The development of thermodynamic models focusing on lactic acid-based chain elongation might shed more light on these competitive pathways.

Modelling thermodynamics only goes so far, and the composition of the microbiome must be considered as this can influence the microbiome's metabolic capacity. The results from microbial community composition analysis using 16s rRNA gene sequencing of the mentioned studies cited above indicate they had a different microbiome structure. The fermentation where the acrylate pathway took over, had a wider variety of prokaryotic families and was dominated by *Acinetobacter* spp. (approx. 60% relative abundance) and the operational taxonomic units belonging to Ruminococcaceae were less than 10% [77].

On the contrary, the study with minimal C3 production was dominated by a Clostridium cluster IV group (79.1%, belonging to Ruminococcaceae) [88].

Table 2-2 Biochemical reactions that compete with chain elongation and their thermodynamic information with concentrations and pressures of all components at 1 M or 1 bar.

Equation	Competitive reactions for chain elongation	$\Delta G_r°$ or $\Delta G_r°'$ [kJ mol^{-1}]	Ref
10.a	Hydrogenotrophic methanogenesis	-125.84[a]	[26]
	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$		
10.b	Acetoclastic methanogenesis	-39.06[a]	[26]
	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$		
11.	Sulphate reduction		[69]
	$CH_3COO^- + SO_4^{-2} \rightarrow H_2S + 2\ H_2O + 2\ CO_2$	-64.39[b]	
	$2\ CH_3CH_2(OH)COO^- + SO_4^{-2} \rightarrow H_2S + CH_3COO^- + 2\ HCO_3^-$	-82.92[b]	
	$2\ CH_3CH_2OH + SO_4^{-2} \rightarrow H_2S + 2\ CH_3COO^- + 2\ H_2O$	-69.29[b]	
	$4\ H_2 + SO_4^{-2} \rightarrow H_2S + 4\ H_2O$	-39.70[b]	
12.a	Lactate reduction to C3: as found in *Selenomonas ruminantium*		[26]
	$CH_3CH_2(OH)COO^- + H_2O \rightarrow CH_3COO^- + CO_2 + 2H_2$ ×1	28.51[a]	
	$CH_3CH_2(OH)COO^- + H_2 \rightarrow CH_3CH_2COO^- + H_2O$ ×2	-86.63[a]	
12.b	Lactate reduction to C3: as determined for *C. propionicum*		[47]
	$CH_3CH_2(OH)COO^- + H_2 \rightarrow CH_3CH_2COO^- + H_2O$	-83.80[b]	
13.	Carboxylate to alcohol reduction with H_2		[12]
	$CH_3COO^- + H^+ + 2H_2 \rightarrow CH_3CH_2OH + H_2O$	-7.22[a]	
	$CH_3CH_2COO^- + H^+ + 2H_2 \rightarrow CH_3(CH_2)_2OH + H_2O$	-7.49[a]	
	$CH_3(CH_2)_2COO^- + H^+ + 2H_2 \rightarrow CH_3(CH_2)_3OH + H_2O$	-3.58[a]	
	$CH_3(CH_2)_4COO^- + H^+ + 2H_2 \rightarrow CH_3(CH_2)_5OH + H_2O$	-7.55[a]	
14.a	Ethanol oxidation: as determined for *C. formicoaceticum*		[47]
	$2\ CH_3CH_2OH + 2CO_2 \rightarrow 3\ CH_3COO^- + 3H^+$	-76.90[b]	
14.b	Coupled ethanol oxidation and C3 reduction		[26]
	$CH_3CH_2OH + H_2O \rightarrow CH_3CHOO^- + H^+ + 2\ H_2$ ×1	7.22[a]	
	$CH_3CH_2COO^- + H^+ + 2\ H_2 \rightarrow CH_3(CH_2)_2OH + H_2O$	-7.49[a]	

[a] at 37 °C, pH 6.82; [b] at 25 °C, pH 7

Studying the microbiome composition improves the understanding of the MMC fermentation mechanisms. For instance, when following the microbial community dynamics of maize silage fermentation in a leach bed reactor (LBR), Sträuber et al. [89] found *Lactobacillus* and *Acetobacter* strains dominated during the first days of operation, with lactic and acetic acid as concurrent products. However, *Clostridium* species became dominant on Days 3 and 4 resulting in a pH increase and C4 and C6 production, and in turn these were overgrown during Days 5 to 7 by other phylotypes capable of using more complex polysaccharides by different metabolism [89]. Further investigation of microbial interactions and synergies will allow better design of MCCA production processes from complex feedstocks, for example operating in sequential batch mode to allow the different trophic groups to first accumulate ethanol, lactic acid, H_2 or VFA for subsequent chain elongation. Only 20 studies on MCCA production could be found, so far, that include an analysis of the microbial community. This usually involves DNA extraction and sequencing of 16s rRNA amplicon and comparison to sequence databases [23, 70, 73, 75, 77, 83, 88, 90-99], sometimes in addition to other community analysis such as flow cytometry [100], analysis of terminal restriction fragment length polymorphisms (T-RFLP) [101] or microscopic evaluation [102]. Recently, Scarborough et al. [103] combined metagenomic, metatranscriptomic and thermodynamic analysis of samples from a reactor microbiome fermenting a lignocellulosic-based feedstock in continuous stirred-tank reactor (CSTR) mode, which allowed, for instance, affiliation of *Lactobacillus* and members of the *Coriobacteriaceae* family to hydrolysis and primary fermentation, and organisms related to *Lachnospiraceae* and *Eubacteriaceae* to MCCA production. In addition, the recent advancements in metagenomic and metatranscriptomic analysis led to the proposition that other MCCA-producing pathways occur in a microbiome, such as the fatty acid biosynthesis pathway, alongside the reverse β-oxidation pathway [104].

Research is necessary to expand the thermodynamic models to include both the MMC composition, which indicates the potential bio-reactions in a system, and the composition of complex feedstocks. The development of these models will complement the understanding obtained from experimental studies, and will help in determining the operational parameters which select for MCCA production over competitive reactions. In addition, culture-independent analysis and increased application of “omics” approaches on MMC fermentation studies will be essential to enhance our understanding of the underlying mechanisms that include competitive and synergistic processes and the importance of the MMC composition.

2.1.4. Bio-waste composition and its effect on chain elongation

A feedstock suitable for chain elongation should provide the necessary substrates, i.e., VFA and electron donors such as ethanol or lactic acid. Chain elongation substrates can either be directly present in the feedstock, indirectly produced from primary fermentation *in vivo*, or supplemented. The highest MCCA production rates obtained in MMC fermentation used a synthetic feedstock, hence a readily bio-available substrate. In up-flow reactors (URs) with biomass retention, 115.2 gCOD $L^{-1}d^{-1}$ for C6 [105] and 19.4 gCOD $L^{-1}d^{-1}$ for C8 [96] were obtained from ethanol and C2 mixtures. These rates are more than 10 times higher than that achieved so far using complex, un-supplemented feedstocks (Table 2-3). If electron donors, such as ethanol or lactic acid, are supplemented, selectivity of secondary fermentation is enhanced towards chain elongation. Ethanol-supplemented organic waste streams have reached production rates that lie somewhat in between synthetic and complex feedstocks. The maximum reported is 60.7 gCOD $L^{-1}d^{-1}$ C6 and 2.13 gCOD $L^{-1}d^{-1}$ C8 for pre-fermented OFMSW supplemented with 97.4 gCOD $L^{-1}d^{-1}$ ethanol [106]. Supplementation of 21.3 gCOD L^{-1} lactic acid to pre-treated grass in batch fermentation with an adapted inoculum resulted in a total C6 concentration of 24.1 gCOD L^{-1} after 1 day [95].

When applying a supplementation strategy, certain experiments show that excessive concentrations of ethanol and lactic acid should be avoided. An upper limit for ethanol-based chain elongation is reported at 97.4 gCOD L^{-1} after which it exerts an inhibitory effect [107]. Grootscholten et al. [108] spiked a LBR processing OFMSW at four intervals with 11.2 gCOD L^{-1} ethanol during batch fermentation. This increased MCCA concentration from 4.0 to 6.0 gCOD L^{-1} for C6 and 0.0 to 1.2 gCOD L^{-1} for C8 compared to a non-supplemented control experiment, yet the total amount of carboxylic acids produced was lower [108]. This was attributed to ethanol inhibition of hydrolytic and acidogenic bacteria [108]. To avoid ethanol inhibition limiting primary fermentation, Grootscholten et al. [106] suggested the use of a two-stage system where hydrolysis and primary fermentation of OFMSW occurs in the first batch phase, and in the second the pre-fermented OFMSW is supplemented with ethanol to select for chain elongation in a CSTR. The disadvantages of two-stage systems are the increased operational complexity and additional capital and operational costs.

Table 2-3 *Overview of feedstock, operating parameters and product outcome (as C6 production and C7 or C8 when data available) in continuous MMC fermentation.*

Feedstock	Reactor (a)	pH	T	OLR	HRT	C6 (C7, C8)		Target	Ref.
		-	°C	gCOD $L^{-1}d^{-1}$	d	gCOD $L^{-1}d^{-1}$	gCOD L^{-1}	product	
Ethanol Rich									
Diluted yeast fermentation beer	SBR (48h)[b]	5.5	30	10.70	15	7.52	NA	MCCA	[37]
Diluted yeast fermentation beer	SBR (48h)	5.5	30	5.70	12	4.62	NA	MCCA	[93]
Diluted wine fermentation residue	UR[b]	5.2	37	51.90	0.9	4.1 (0.3, 2.5)	NA	MCCA	[97]
Yeast fermentation beer and thin stillage	SBR (24h)	5.5	35	2.89	7	2.55	17.90	MCCA	[92]
Yeast fermentation beer	SBR (48h)	6.5	30	7.64	15	1.74	NA	MCCA	[27]
Syngas fermentation effluent, with nutrients	UR[b]	5.5	30	51.80	0.58	3.80	2.25	MCCA	[110]
Lactic Acid Rich									
Acid whey from quark industry	UR[b]	5.5	30	28.80	2.5	5.12	NA	MCCA	[100]
Pre-fermented acid whey yoghurt industry	UR[b,ex]	5.0	30	10.90	2.1	3.08	<1.00	MCCA	[94]
Diluted yellow water	USBR (67.2h)	6.0	30	8.67	28	1.85	51.70	MCCA	[88]
Diluted cheese whey powder	UR[b]	6.0	37	5.36	4	0.70	2.80	VFA	[111]
Cheese whey	SBR (24h)	NA	35	6.00	12	0.12*	1.45*	VFA	[123]
(Ligno) Cellulosic Based									
Switchgrass-derived stillage	CSTR	5.5	35	7.20	2	5.74 (NA, 0.66)	18.70 (NA, 2.40)	MCCA	[99]
Pre-fermented grass	SBR (24h)	NA	32	5.30	2	4.52	9.03	MCCA	[95]
Corn-derived thin stillage	SBR (48h)[ex]	5.4-5.7	35	18.00	3	1.14 (0.10, 0.00)	NA	MCCA	[23]
Pre-treated corn fibre: dilute-acid/ dilute-alkali/ hot water	SBR (48h)	5.5	55	1.92	15	0.6* / 0.39* / 0.12*	NA	VFA	[36]
Paper fines and industrial bio-sludge (40:60)	SBR[a]	NA	40	2.6 gVS L^{-1}	16	0.42 (0.08, NA)	6.67 (1.36, NA)	MCCA	[124]
Food Waste									
Simulated food waste	SBR (24h)	5-6	34	30 gTS L^{-1}	3	8.09*	24.30*	H_2	[116]
Restaurant food waste	LBR (7d)	NA	37	16.60	7	3.12	21.80	MCCA	[91]
Cafeteria food waste	SBR (24h)	5.5	45	9 gTS L^{-1}	8	1.28	10.30	VFA	[125]
Vegetable and salad waste	SBR (24h)	5-7.5	35	0.57	20	0.25*	5.08*	VFA	[126]
Other									
Sucrose-rich synthetic wastewater	UR	3.6	35	15.80	0.71	5.70	4.03	H_2	[115]
Synthetic glucose medium	UR[b]	NA	30	204.00	0.10	4.13*	0.43*	H_2	[127]
Primary and waste activated sludge	SBR (24h)	NA	35	79.90	1	< 0.08*	< 0.08*	VFA	[123]
Food Waste with Ethanol Supplementation									
Pre-fermented OFMSW, 44.8 gCOD L^{-1} ethanol, CO_2	UR[b]	6.75	30	NA	0.46	60.70 (4.59, 2.13)	27.80 (2.10, 0.98)	MCCA	[106]
Pre-fermented food waste, 78.4 gCOD L^{-1} ethanol, CO_2	CSTR[b]	6.8	30	28.90	4	12.80	51.20	MCCA	[128]
Pre-fermented food waste, 78.4 gCOD L^{-1} ethanol, CO_2	CSTR[b]	6.8	30	115.00	1	12.40 (0.33, 0.42)	15.70 (0.18, 0.18)	MCCA	[128]

SBR: sequential batch reactor; UR: up-flow reactor; CSTR: continuous stirred tank reactor; NA: not applicable; (a) fermentation cycle in case of SBR or semi-batch; [b] biomass retention; [ex] *in situ* extraction; * estimated from graphs. When values were not directly reported by cited study these were estimated as described in Supplementary.

A life-cycle assessment on C6 production from ethanol-supplemented food waste fermentation in a lab- and pilot-scale system revealed that the largest environmental effects (acidification and eutrophication potential) result from addition of caustic soda to control pH and ethanol as electron donor [109]. Thus, supplementation of electron donors to stimulate chain elongation should be minimized or avoided. Instead of supplementing the feedstock, several studies have split the overall fermentation into two stages. Firstly, specific operational conditions are selected to accumulate ethanol or lactic acid. Then in a second phase, the leftover organics in the effluent are fermented towards VFA and elongated with the electron donor under chain elongating conditions. Ethanol-rich substrates fed into chain elongation reactors have been obtained from yeast-based fermentations, such as for the production of bio-ethanol which generates an ethanol-rich beer and a residue after distillation called stillage [27, 37, 93], or residues from the production of wine [97], or effluent from syngas fermentation [110]. Lactate-rich substrates can be obtained via MMC fermentations selective for lactic acid, such as effluent from thermophilic acid whey fermentation [94], and pre-fermented grass (grass silage) [95] or maize silage [27, 89]. Whey is rich in lactose, simple sugars such as fructose that are easily fermented to produce lactic acid [111]. Food waste contains lactic acid bacteria that are easily enriched in MMC fermentation, to produce lactic acid up to concentrations of 21.3 to 48 gCOD L^{-1} [112-114]. Maximum production using ethanol- and lactic acid-rich streams in MMC fermentation without additional supplementation have been reached using diluted beer (7.52 gCOD $L^{-1}d^{-1}$ C6) [46] and acidified whey from the quark industry (5.12 gCOD $L^{-1}d^{-1}$ C6) [85]. The disadvantages of two-stage systems for accumulation of electron donors are the increased operational complexity and additional capital and operational costs.

An alternative to supplemented or pre-fermented organic feedstock, would be to operate a single-phase process where the substrate itself is converted to lactic acid and/or ethanol in parallel with the chain elongation reactions. Recently, C6 has been produced in a one-stage LBR from food waste up to concentrations of 21.8 gCOD L^{-1} and a rate of 3.12 gCOD $L^{-1}d^{-1}$ [91], comparable to a supplemented and/or two-stage system. In this study, batch tests inoculated with the LBR leachate showed that lactic acid was consumed in favour of ethanol as the electron donor [91]. Other studies aimed at VFA or H_2 production have produced C6 in a single-stage approach from food waste, without supplementation, and at similar production rates. In studies aimed at H_2 production using glucose-rich synthetic wastewater [115] or simulated food waste [116] as feedstock, C6 was produced at rates higher than experiments which targeted MCCA production (Table 2-3). Therefore food waste and similar complex substrates might be a promising feedstock.

The use of complex feedstock comes with a set of challenges. Firstly, the composition of the feedstock will influence certain competitive processes and the intermediates that result from hydrolysis and acidogenesis, and therefore affects the potential for chain elongation. For example, VFA yields in MMC fermentation have shown to be less for lipid-rich waste compared to carbohydrate- or protein-rich wastes, due to difficulties in hydrolysis [117]. In addition, the type of carbohydrates [118] or proteins [119] in the substrate also influences the metabolic pathways. Secondly, it is important to recognise that seasonal and geographical variation in organic waste streams impacts composition and availability of feedstock. This can often become a challenge when designing a suitable treatment or valorisation (bio-)process that is robust and flexible, especially when relying on pure microbial cultures. For instance, OFMSW may contain small quantities of bio-waste from various origins, such as discarded oils, fruits, animal-derived products and more lignocellulose-rich leaves and stems from vegetables, resulting in an overall feedstock comprising both easily biodegradable carbohydrates and proteins as more recalcitrant matter [120]. It was found that a LBR fed with OFMSW which was richer in green waste had lower production of carboxylic acids compared to OFMSW containing more food waste [121]. Lastly, not every type of substrate will allow chain elongation to occur. In batch experiments aimed at VFA production, seven types of “food waste” feedstocks were tested and only four produced C6. These were cheese whey, sugarcane molasses, the organic fraction of municipal solid waste, and winery effluent [122]. Similarly, in sequential batch reactors (SBRs), the fermentation of cheese whey resulted in 0.12 gCOD $L^{-1}d^{-1}$ C6, while in similar operating conditions the fermentation of sewage sludge produced more total carboxylic acids but very little C6 [123]. Therefore, the right type of complex feedstock should be selected for MCCA production to be successful – this is not well understood and requires further investigation.

2.1.5. Environmental conditions that influence chain elongation

2.1.5.1. Mesophilic temperatures seem to benefit chain elongation

Temperature has a significant influence on energy released from reactions [80], alters the microbial community composition [129], and affects the kinetic rates of metabolic reactions. The amount of research focusing on the effect of temperature on chain elongation is limited, yet results so far indicate operation in the mesophilic range, typically between 30 °C and 45 °C, is preferred over thermophilic conditions. In a long term experiment fermenting ethanol-rich beer (from a yeast fermentation) in a SBR with in-line product extraction, selectivity for C6 nearly tripled after reducing the temperature from 55 to 30 °C [130].

The effect of temperature on acidogenesis and VFA production has been studied in more depth and could provide further insight. In a study evaluating acidogenesis of cafeteria food waste, trials at 25, 35 and 45 °C gave the highest overall carboxylic acid yield at 35 °C (~23.5 g L^{-1} total carboxylic acids with ~3.1% C6), yet the C6 yield was higher at 45 °C (~19.8 g L^{-1} total carboxylic acids with 24% C6) [125]. VFA-targeted fermentation of pre-treated cellulosic biomass with meso- (40 °C) or thermophilic (55 °C) operation showed a significant divergence in the proteins and enzymes present and in MMC composition. Mesophilic operation allowed C6 formation to occur (3 to 4 % of carboxylic acids) while no C6 was found under thermophilic conditions [129, 131]. The thermophilic reactor maintained a community similar to the inoculum, rich in Clostridia, while the mesophilic system showed a wider variety of taxa which was dominated by members in the Bacteroidetes phylum [129]. While there are some indications that a mesophilic temperature favours chain elongation, more evidence is needed to understand the processes that affect chain elongation productivity at different temperatures. These could be attributed to changes in the collective microbiome metabolism and/or composition, as well as to thermodynamic and kinetic shifts to competing reactions.

2.1.5.2. The influence of pH and buffer capacity on MCCA production

Metabolic pathways, hydrolysis, thermodynamics and, product outcome are significantly influenced by pH in MMC fermentation [123]. For example, in fermentation of cheese whey poor control of pH led to fluctuating carboxylic acid concentrations and hindered establishment of steady state [111]. The specific effects of pH on chain elongation are not straightforward and various factors must be considered.

Firstly, pH determines the dissociation/association equilibrium between carboxylic acids and carboxylates, and therefore the impact of product toxicity due to the acidic form. Secondly, pH determines the CO_2 and carbonate distributions, which affects buffer capacity, and its availability to certain organisms. For instance *C. kluyveri* requires incorporation of CO_2 in its biomass [132]. Thirdly, thermodynamic feasibility depends on pH. Indeed, chain elongation is less thermodynamically favourable under alkaline conditions [133]. Lastly, the effect on the microbiome composition and microbial competition has to be taken into account. For instance, the highest reported production rate of C6 and C8 was obtained at neutral pH, yet a specific methanogenic inhibitor had to be added since acetoclastic methanogens compete under these conditions, and consume C2 for CH_4 production, reducing its availability for chain elongation [105]. Methanogenesis occurs within a pH range of 6.0-8.3 while acidogenic bacteria can tolerate lower pH [134]. Thus a strategy to minimize

CH_4 production is to operate at slight acidic pH (5.0-5.5), and this has been applied in various VFA or MCCA producing experiments [100, 123, 130].

The effects of pH on primary fermentation also have to be considered. Experimental studies have shown that controlling pH at alkaline or neutral values improved liquefaction and hydrolysis of solids in organic waste, but it did not support chain elongation since short chain carboxylates were accumulated without production of MCCA [126, 135-137]. In addition, it is necessary to consider the cost of pH correction, as for example, food waste has an average pH of 5.1 [138]. Operating at neutral or alkaline pH would require substantial addition of pH-correcting chemicals, and hence lowering the economic feasibility [89].

Despite the complex effects of pH on MMC fermentation, some general effects on product outcome are reported for organic or synthetic waste fermentation. A shift to lactic acid or ethanol production was observed in two separate studies, one at pH < 3.6 [115] and another at pH < 4.5 [139]. At slightly higher pH range of 5.0 to 5.5 C3 accumulated as main fermentation product [139]. Two studies have investigated the effect of pH on H_2 production, an important element necessary for chain elongation, which found different results: one suggested optimal production around a pH of 4 [115] and another at pH of 8 [140]. Finally, MCCA production can occur under slightly acidic conditions (5 < pH < 6), although isolates of MCCA producers such as *C. kluyveri* operate best at near-neutral pH [106]. Therefore, in order to favour chain elongation the evidence so far suggests that whilst the target microbial communities might operate across a broader pH range, a slightly acidic or neutral pH might result in the best compromise between thermodynamics, kinetics and microbial competition. The pH might need optimisation depending on other operational parameters and fermentation characteristics, such as other factors influencing competitive reactions or substrate composition.

2.1.5.3. The influence of pH on product toxicity

Another aspect linked with operational pH is product toxicity from C6 and C8 compounds. Carboxylic acids up to a carbon chain length of 18 are potent antimicrobial compounds that interact with lipid bilayers of cell membranes, disrupting the energy generating mechanisms, internal pH, and cell integrity [141, 142]. At acidic pH a higher percentage of MCCA are present in their un-dissociated, toxic acid form. With a pKa of 4.85 for C6 and 4.89 for C8, 50% of the total carboxylate is found in the acidic form at a pH equal to the pKa. C6 and C8 showed similar toxicity in *E. coli* at 10.2 to 14.1 gCOD L^{-1} at pH 7 [143]. For mixed culture chain elongation, different inhibitory concentrations have been reported. Angenent et al. [58] observed the inhibitory concentration of protonated C6 to be 1.92 gCOD L^{-1} in a system using yeast-fermentation beer. Khor et al. [95] obtained stable C6 production around pH 5.5

at just below this limit during fermentation of grass silage with adapted MMC; however, supplementation of lactic acid led to accumulation of protonated C6 concentrations up until 4.4 gCOD L^{-1}, with total C6 concentrations close to the solubility limit of C6 (≈22.7 gCOD L^{-1}).

Table 2-4 *Reported environmental conditions to select for chain elongation in MMC fermentation for MCCA production.*

	Summarized Literature Finding	Ref.
	Temperature	
Preference	Mesophilic range from 30 to 45 °C	[49, 125, 129-131]
Effect	Indications of influence on microbiome composition.	[129]
	pH	
Preference	Slight acidic: preferred range from pH 5 to 6	[100, 123, 130, 134]
	Neutral pH, if specific methanogen inhibitors added	[105, 106]
Self-regulation	Without control, pH usually stabilises between 5.5 and 6.7	[90, 95, 135]
Product toxicity	Protonated C6 toxicity limit: 1.92 gCOD L^{-1}	[37, 58]
	C6 accumulation over toxicity limits is possible (in batch, one point measurement)	[95]
	Alkalinity	
MCCA	No data available	
AD	1 to 5 g_{CaCO_3} L^{-1}	[144, 145]
H_2	0.11 g_{CaCO_3} L^{-1}	[146]
VFA	2.4 to above 30 g_{CaCO_3} L^{-1}	[122, 147, 148]

2.1.5.4. Buffering capacity and potential of self-regulation of pH

The pH in MMC fermentation quickly drops to minimum of 3.0 due to acidification if there is limited buffer capacity or no continuous pH control [140, 149]. The buffering capacity or alkalinity of the fermentation broth is the capacity to neutralize acids via the presence of compounds such as phosphate, bicarbonate, carbonate and hydroxides. Fermentation of Brewer's Spent Grain was shown to reduce pH from 6.5 to 3.8 after only 1 day due to lactic acid production [133]. Also ammonium (NH_4^+) released during hydrolysis of nitrogen-rich feedstock neutralises acids during fermentation by anaerobic consortia [150]. Sometimes chain elongation experiments include a buffer in their synthetic medium [127]; alternatively bicarbonate is added as nutrient requirement for *C. kluyveri* [72, 74, 110].

No studies could be found that evaluate the effect of alkalinity on chain elongation. However, for other types of MMC fermentation alkalinity and buffering mechanisms have been studied. Examples include AD, H_2 production or acidogenesis for VFA production,

which sometimes include the reporting of C6. Alkalinity in MMC fermentation in AD is well studied and its measurement serves as a tool to indicate early warning of unwanted acidification and pH destabilisation [151]. The optimal value of alkalinity usually lies between 1.0 – 5.0 g_{CaCO_3} L^{-1} [144, 145]. For H_2 production by MMC from organic waste, an alkalinity optimum was found around 0.11 g_{CaCO_3} $gCOD^{-1}$ with excessive alkalinity resulting in increased osmotic pressure [146]. Improved VFA production has been noted for fermentation reactors processing cheese whey, the organic fraction of municipal solid waste or synthetic soft drink wastewater with higher alkalinity ranging from 2.4 to above 30 g_{CaCO_3} L^{-1} [122, 147, 148]. Comparison of buffered (pH≈7) and unbuffered (pH drop to approx. 6) batch fermentation of vegetable and salad waste over 70 days showed that addition of ~13.3 g L^{-1} $NaHCO_3$ tripled C6 concentrations for the first 52 days to a maximum of 6.6 gCOD L^{-1}. However, over longer operating times production decreased in the buffered system due to methanogenesis resulting in similar C6 yield for the two systems at the end of operation [126]. Therefore, there are indications that a minimum alkalinity would benefit MCCA production, but this needs to be further investigated.

Studies aimed at VFA production from complex substrates indicate a self-regulation of pH in MMC fermentation in the range of 5.2 to 6.7 [135]. A similar pH stabilisation effect has been noted by studies directed at MCCA formation. For example, batch fermentation of food waste generated a pH drop from 7 to ~5.3 due to hydrolysis and primary fermentation; after 20 days it rose again to pH 5.8 with production of MCCA [90]. For continuous processes this effect results in an overall pH stabilisation. A stable pH between 5.5 and 6.2 occured while feeding a lactic-rich substrate at pH 4.5 to 5 [95]. Chain elongation via lactic acid consumes protons (Table 2-1, Eq. 7-9), although no studies have investigated the link between lactic acid-mediated elongation and the buffering of pH. Further studies are needed to show the minimal alkalinity required for chain elongation in MMC to sustain a minimum buffer capacity, which also allows continuous operation at optimal pH, and with limited or no pH control.

2.1.6. The push towards chain elongation by organic overloading

For AD applications, organic overloading is defined as the COD loading rate exceeding the degradation capacity of the anaerobic microbiome, leading to accumulation of VFA and hence a decrease of pH which inhibits methanogens [152]. For VFA and MCCA production, organic overloading is deliberately employed to promote carboxylic acid accumulation and limit competitive processes. Elevated carboxylic acid concentrations inhibit methanogens, as found, for instance, for C4 where only ~2.4 gCOD L^{-1} at pH 6 was shown to inhibit 90% of methanogens in a thermophilic batch fermentation [130].

The organic load at start-up can be represented by the food-to-microorganisms ratio (F/M). This is defined as the amount of feedstock introduced, expressed as COD or volatile solids (VS), relative to the amount of biomass, estimated as VS or volatile suspended solids (VSS) in the inoculum [9, 153]. In anaerobic consortia fermenting food waste, organic overloading and VFA accumulation usually occur at start-up with a F/M ratio > 1 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$ and carboxylates accumulate at an optimal F/M ratio of 5 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$ [9]. As with the acidogenic fermentation of synthetic soft drink wastewater, a F/M ratio of 4.0 $gCOD_{fed}$ $gVSS_{inoculum}^{-1}$ was found to be optimum for C6 production compared to F/M ratios of either 1.6 and 6.4 $gCOD_{fed}$ $gVSS_{inoculum}^{-1}$ [148].

A positive relationship is found for MCCA production and higher organic loading rates (OLRs) using synthetic or supplemented substrates and pure cultures or MMC [96]. However, for complex feedstock, whilst the same mechanism would be expected, the relationship between OLR and MCCA production is less straightforward (Figure 2-3). When using a synthetic medium or supplementation of electron donors the OLR, expressed in terms of total COD, is directly related to the amount of bio-available substrate. On the contrary, when using complex feedstock, the OLR does not necessarily indicate the presence of chain elongation substrates or anaerobic biodegradable content, which can be converted to chain elongation compounds. Namely, fractions can be present in complex feedstocks that can be chemically oxidised, and thus contribute to the total COD, but are not easily biologically degraded. For instance, bio-waste collected from municipalities had a higher anaerobic biodegradability (90.8 ± 3.7 %) compared to food waste collected from a vegetarian restaurant (66.9 ± 6.4 %) even though their total COD values were within a similar range, i.e., 337 ± 14 gCOD kg_{ww}^{-1} and 303 ± 18 gCOD kg_{ww}^{-1} respectively [17]. MMC fermentation experiments for MCCA products using complex feedstocks have only recently been conducted, and little is known regarding the need for easily biodegradable feedstock in order to exert sufficient OLR to accumulate chain elongation substrates for C6 production. In AD, high-COD substrates such as food waste or food processing by-products result in problematic increased retention times and low throughput, to prevent organic overloading and hence such feedstocks show greater potential for production of MCCA rather than biogas.

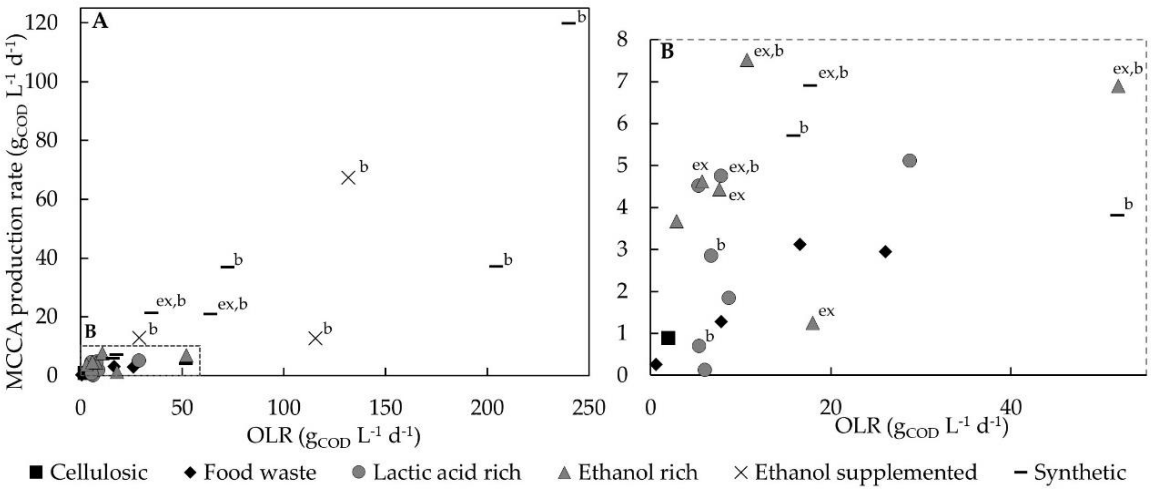


Figure 2-3 *The highest reported MCCA (C6 to C9) production rate as a function of OLR and type of feedstock from 27 different studies using (semi-)continuous MMC fermentation towards MCCA, VFA or H_2. Experiments including in situ product removal or biomass retention are marked with "ex" or "b", respectively. Studies using complex feedstock are only included which report production rates and OLR in gCOD L^{-1}, or where such values can be estimated using calculations described in Supplementary. Studies using synthetic substrates only include those listed in Table 2-3 and three additional studies which used synthetic media comprising ethanol and C2 to represent highest reported rates with HRT optimisation [72],[105] and in situ extraction [96] respectively. Data collected from [23, 27, 36, 37, 72, 77, 88, 91-97, 100, 105, 106, 110, 111, 115, 116, 123, 125-128, 154], the full data for the figure can be consulted in the following database [55].*

2.1.7. Effect of inoculum on achieving MCCA

Selection of the appropriate inoculum in biotechnological applications is critical to ensure catalysis of the required bio-conversion process. Cultures used for inoculating reactor experiments with complex substrates for carboxylate production contain a large variety of microorganisms (high richness) and include anaerobic sludges from AD [147] or wastewater treatment [133], marine sediments [107, 129], rumen samples [30, 40], mixtures of cultures [155] or microbiomes enriched in chain elongators obtained from lab-scale reactors [83, 91, 96]. For production of VFA, H_2 or other MMC fermentation products, the inoculum can be physico-chemically pretreated in order to suppress methanogenesis, e.g., heat shock and/or acid/alkali conditioning [156, 157]. However, no studies could be found on the effect of inoculum pretreatment on MCCA production specifically. Studies on MMC fermentation towards H_2 or VFA have shown that the microbial composition was altered and the MMC fermentation product profile shifted depending on inoculum pretreatment method applied [7, 47, 158, 159]. For chain elongation in particular, Cavalcante et al. [69] has discussed thermal pretreatment as a potential selective pressure since various chain elongating bacteria have been allocated to the genus *Clostridium*, that due to its spore-forming abilities could be selected for by heat shock. The limited knowledge regarding pretreatment of inoculum to select for chain elongation would be worthwhile to further investigate.

Separate inoculation is not always required. Some experiments showed C6 production from organic waste by simply using the endogenous MMC present in the feedstock [108, 135].

In addition, regardless of the initial inoculum source (i.e., sludge from full-scale AD plant or lab-scale fermentation reactors) microbiomes grown on synthetic substrates with ethanol as the main electron donor are enriched by species closely related to *C. kluyveri* due to adaptation over time [70, 101].

As shown in Figure 2-4, for batch experiments, the most commonly used inoculum types are AD sludge or enriched microbiomes, i.e., previously enriched and adapted to chain elongation conditions in the lab. Yields seem to be similar for both types of inoculum, perhaps due to high microbial richness. However, the total MCCA concentration accumulated with an AD inoculum had a 5 times lower upper range than when an adapted microbiome is used, indicating that tolerance towards toxic MCCA concentrations can be developed (Figure 2-4). This higher tolerance to higher C6 concentrations from acclimatised microbiomes has been recently reported using batch inhibition assays with synthetic media and differently cultured sludges [160]. The same adaptability hypothesis was used to explain productions of longer MCCA such as C8 [92]. However, batch experiments inoculated with an enriched microbiome are often operated at a higher organic load that could also enhance MCCA accumulation, and these parameters are inter-connected. From the analysed studies it also appears that bio-augmentation does not improve considerably the MCCA yields, but it does impact on the product concentration with more MCCA produced. It has been shown that bio-augmentation with *C. kluyveri* improves yields and even results in chain elongation up to decanoic acid (C10) [90]. From the data currently available, inoculum selection could contribute to MCCA specificity, yet further investigations are needed to evaluate the importance of inoculum selection.

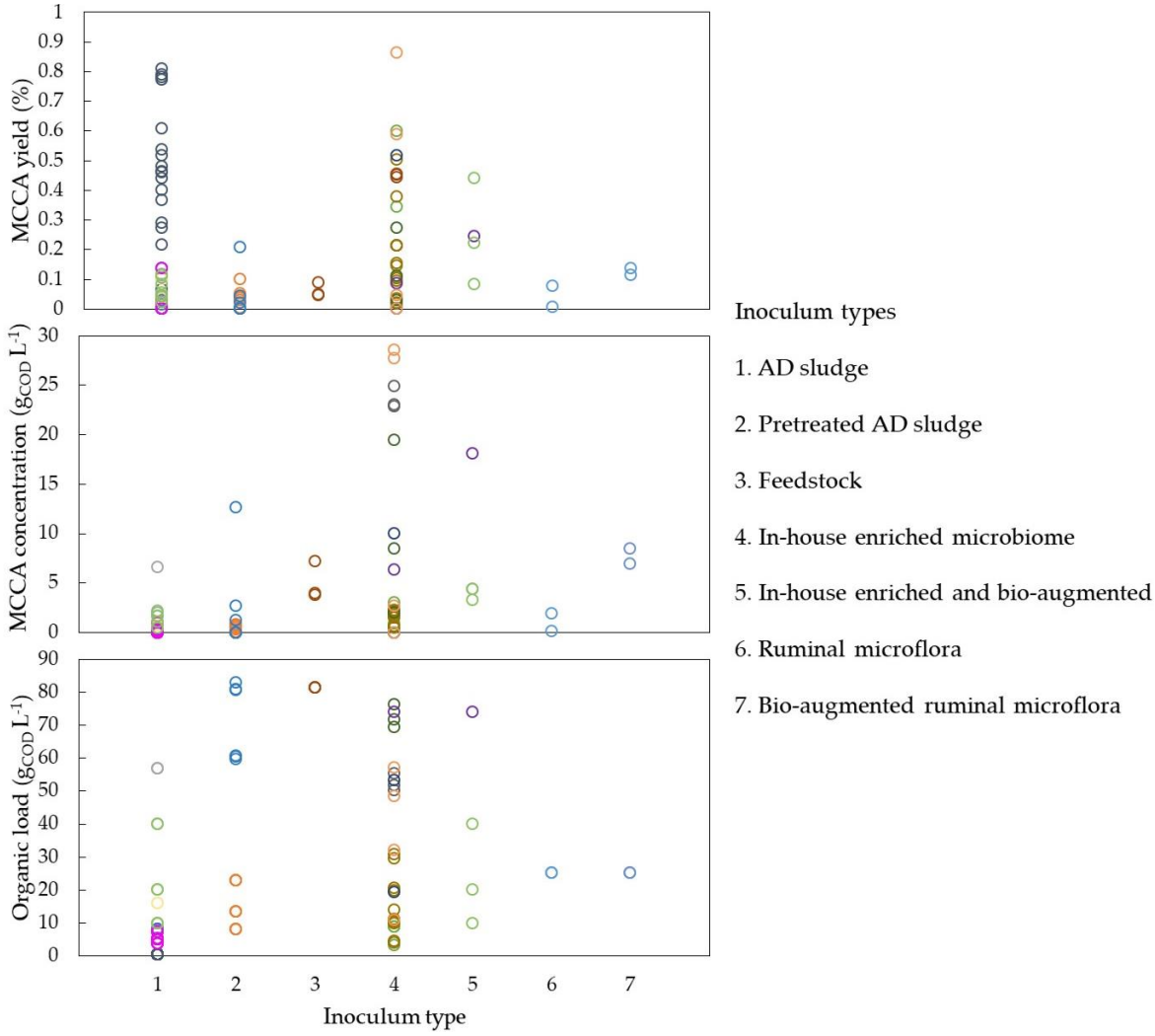


Figure 2-4 *Inoculum type used in 17 studies performing batch experiments of complex or synthetic feedstock in function of the obtained MCCA yield (top), MCCA concentration (middle), or organic load applied (bottom). Colours represent experimental results reported in the same study. Estimated values were calculated as reported in Appendix A. Data collected from [20,40,70,83,88,90,91,96,101,108,122,126,148,157,161-163], the full data for the figure can be consulted in the following database [55].*

2.1.8. The influence of partial pressures in reactor headspace

2.1.8.1. The aerotolerance of MMC fermentation

M. elsdenii and *C. kluyveri*, two well-characterised bacteria capable of chain elongation, are both strictly anaerobic [61, 132]. Anaerobic conditions are easily maintained in laboratory experiments by flushing the headspace with N_2, CO_2 or H_2 or mixtures thereof. However, full-scale or pilot reactors are not always operated fully anaerobically [161]. Therefore, it is important to understand the sensitivity to the presence of oxygen on MMC when producing MCCA.

In a study investigating the aerotolerance of MMC fermentation of shredded paper and chicken manure, intermittent air exposure had no significant influence on bacterial

community composition, however, it did select for shorter chain carboxylates, whilst stricter anaerobic conditions improved chain elongation [161]. On the contrary, in another study using different types of pre-treated corn fibre, air exposure did not lower the C6 production rate [36]. There is still very little evidence of the capacity of MMC cultures performing chain elongation in regards to their tolerance of oxygen and its impact on the production of MCCA. This should be the subject of further studies as it is an important design parameter for experimental and full-scale chain elongation processes.

2.1.8.2. Minimal and balanced CO_2 and H_2 required for chain elongation

A sufficiently high H_2 partial pressure (pH_2) in the headspace gas is an important parameter to limit the impact of competitive processes and is seen as a central strategy to ensure reductive conditions for reverse β-oxidation [130]. However, the mechanism of how pH_2 affects chain elongation is not fully understood, and various values have been reported (Table 2-5). Similarly, pH_2 controls the VFA yielding of anaerobic MMC fermentation such as AD. Different pH_2 have been reported to inhibit or thermodynamically constrain certain bioconversion processes thereby influencing the VFA product spectrum [162, 163].

Table 2-5 *Experimental and theoretical reported values of pH_2 and/or pCO_2 in reactor headspace to stimulate chain elongation in MMC fermentation.*

Headspace requirement	Influence on Chain Elongation	Ref
	Hydrogen	
pH_2 < 0.1 bar	Theoretical maximum pH_2 for oxidation of ethanol to C2 for ATP generation to be thermodynamically feasible	[58]
pH_2 < 1.5 bar	Experimental pH_2 minimum for reduction of C2, C3 and C4 to alcohols (pH = 5)	[165]
pH_2 = 1.5 bar	Initiated production of C6 from ethanol and C2 in experiment	[70]
pH_2 > 2.52 ×10^{-6} bar	Theoretical minimum pH_2 to prevent oxidation of C6 at experimental concentrations	[37]
0.03 bar < pH_2 < 1.5 bar	Recommended pH_2 for chain elongation	[106]
	Carbon dioxide	
CO_2 or CO_3^{2-} addition	Lowered pH, improved MCCA production	[105]
	Mixture of Hydrogen and Carbon Dioxide	
pH_2 ≈ 0.007% by dosing CO_2	Excess oxidation is thermodynamically prevented	[98]
H_2/CO_2 = 60/40	MCCA produced from H_2/CO_2 mixture	[166]
H_2/CO_2 = 80/20 at 0.5 bar	Enhanced production of C6 from ethanol and C2 in experiment	[91]
pH_2 = 2 bar, pCO_2 = 2 bar	Reduced C3 formation	[117]
pH_2 = 1 bar, pCO_2 = 0.3 bar	Optimal thermodynamics for chain elongation	[30]

In thermodynamic fermentation models, it is assumed that dissolved H_2 affects the NADH/NAD^+ ratio directly, and hence the thermodynamic feasibility of certain pathways [81]. Nevertheless, experimental work could not find a direct correlation between the two, thus possibly indicating a more complex effect where alternative electron carriers (e.g., ferrodoxin) are involved in MMC [164]. H_2 is a product from ethanol- and lactic acid oxidation occurring during chain elongation [29], yet it is also an indirect electron donor for chain elongation by its capability of reducing C2 to ethanol [127]. A minimum pH_2 (a composition > 0.007% at standard conditions) was found to prevent excessive oxidation of ethanol (i.e., ethanol oxidation to C2 not coupled to chain elongation) or oxidation of carboxylates [37, 98]. In batch microbiome studies using synthetic C2-rich media, C6 and C8 were only produced in the presence of H_2, even in the absence of ethanol [70, 91]. If pH_2 is too high, carboxylates are reduced to their corresponding alcohol [165]. In addition, a pH_2 above ~0.1 bar reduces the thermodynamic favourability of ethanol oxidation to C2 for ATP generation [58, 81]. In general, it is stated that the pH_2 should be above ~0.03 bar to avoid excessive ethanol oxidation to C2 while remaining below ~1.5 bar to prevent carboxylate reduction [106].

Another important headspace component is CO_2. With a MMC membrane biofilm reactor MCCA were produced solely from a 40/60 ratio of CO_2 and H_2 [166]. CO_2 is also a nutritional requirement for some chain elongating bacteria [132]. In addition, CO_2 partial pressure influences dissolved carbonate and thus the alkalinity. Experimental studies have shown CO_2 addition in the headspace improved chain elongation [105], and a combination of CO_2 and H_2 in the reactor headspace reduced C3 formation [117]. CO_2 dosing within the reactor headspace was recently proposed as a key strategy in controlling ethanol-based chain elongation, where high CO_2 loading rates for ethanol-rich feedstock could stimulate excessive ethanol oxidation to C2, and low values for VFA-rich (and low-ethanol) feedstock could ensure ethanol is used in chain elongation and not for C2 production [98]. Dosing of CO_2 is inversely proportional to the pH_2, thus care must be taken to ensure minimal pH_2. Weimer et al. [30] calculated the H_2:CO_2 ratio that shows the optimal thermodynamics for chain elongation and suggested approx. 1 bar pH_2 with 0.3 bar pCO_2.

Using a complex feedstock will lead to production of CO_2 and H_2, since they are both products of primary fermentation by various hydrolytic and acidogenic microorganisms [167]. Therefore, modifying the headspace composition by allowing these gases to accumulate in batch operation in closed vessels [83], working with pressure release in reactors [133] or intermittent opening of reactor headspace for sampling [148], is expected to affect chain elongation. This effect is usually not taken into account in experiments, and to our current knowledge, no studies have specifically assessed the effect of accumulation

of these gases in the reactor headspace during primary fermentation on chain elongation. The partial pressure of gases in the reactor headspace should be considered in the design and operation of reactors as plenty of studies demonstrate their influence on chain elongation. However, it must be noted that H_2 is a highly soluble gas and its concentration in the liquid can be up to 70 times higher than the equilibrium value suggested from mass-transfer coefficients in AD [168]. Therefore, care must be taken to relate pH_2 with H_2 available for bioconversion processes.

2.1.9. Reactor Design and the Relation to Retention and Organic Overload

The type of feedstock will influence the choice of fermentation reactor [8]. In the case of a complex, solid-rich feedstock, such as OFMSW or unprocessed food waste, hydrolysis is rate limiting and stirring or pumping is impractical. Therefore, longer retention times are required to allow hydrolysis and solubilisation, and leach bed reactors (LBRs) are an effective option, as they generate a leachate rich in carboxylic acids. For instance, Yesil et al. [169] obtained ± 30 gCOD of carboxylic acids per kg of solid waste of which approx. 10% was C6, in batch LBR. Nzeteu et al. [91] used a LBR set-up that allowed semi-continuous operation and obtained a maximum C6 production rate (3.12 gCOD $L^{-1}d^{-1}$) from food waste by replacing 75% of the reactor content with fresh feedstock and diluting the leachate with water by 1/15 every 7 days. However, this approach does not easily allow operation with homogenous pH or temperature stability. Another study has shown that operation stability for the production of H_2 by MMC fermentation of food waste is enhanced by mixing and agitation [116], however, this has not yet been determined for chain elongation.

To exert better process control, complex feedstock can be mechanically pre-treated, i.e., crushing, chopping or blending, to obtain a mixture that can be pumped and stirred, and therefore allows the use of (semi-)continuous stirred tank reactors (sCSTRs). This has been done in some studies focussing on carboxylic acid production from food waste [125, 126]. In addition, the feed stream can be diluted with water, or blended with a liquid waste stream or recycled liquor from sludge dewatering to modify the composition. For a more fluid stream such as synthetic feedstock, e.g., ethanol and acetate mixtures, or more easily degradable substrates such as potato-processing or brewery wastewater or food waste leachate, less hydrolysis is required and the chain elongation itself becomes rate-limiting. To allow higher flow rates, whilst maintaining high biomass to counter the rate-liming effect of chain elongation, reactor configurations such as membrane bioreactors [166], CSTRs with *in situ* settlers [128] and up-flow reactors (UR) with mechanisms for biomass retention, e.g., filter, sludge blankets or packed beds, have been used (Table 2-3). It has been suggested that

biomass retention and cell density are important parameters which are often unreported for MMC fermentation [95]. Indeed, studies using reactors with biomass retention have shown the highest MCCA production rates so far (Figure 2-3). Such reactors are well established for other processes, and are worthy of investigation.

In continuous systems, the inoculum acclimation and biomass retention, also expressed as sludge retention time (SRT), favour specific microbial populations. For instance, C4 producers have often been shown to have a longer doubling time than lactic acid producers, and hence wash out more easily from continuous systems [47]. In stirred tank reactors without a settling phase, the SRT is equal to the hydraulic retention time (HRT) and inversely proportional to the OLR. Therefore, in order to operate at a sufficient biomass retention, the OLR can only increase with an increase of substrate COD composition. A suboptimal residence time results in lactic acid or VFA accumulation without chain elongation, and a reduction in hydrolysis [97, 128]. In fermentation of salad and vegetable waste in a sCSTR, a retention time of 10 days left a fraction of the substrate unutilized and no C6 was produced; whilst retention time of 20 and 30 days increased C6 production [126]. Similarly, a step-wise decrease of HRT from 20 to 12 to 8 days in cheese whey fermentation, generated C6 concentrations which decreased from 2.24 gCOD L^{-1} to 1.45 gCOD L^{-1} to 0.41 gCOD L^{-1}. Analysis of the microbiome composition revealed certain microbial groups were removed by lowering the HRT, via washout, with a dominant presence of lactic acid-producing *Lactobacillus sp*. at HRT of 8 days [123]. Thus, a minimum HRT is required to sustain chain elongation when working with reactors without biomass retention. However, if the HRT is too high competing processes such as methane production are more favoured [36, 126]. Methanogens are relatively slow growers, thus reducing HRT has been suggested as a tactic to wash them out, and increase MCCA production rates [72, 106]. Therefore, a compromise to reduce methanogens and enhance chain elongation must be found.

Studies on chain elongation have varied HRT from less than 1 day to over 2 weeks resulting in various MCCA production yields (Figure 2-5). A lower HRT can generate similar yields by altering reactor design to include biomass retention and hence decoupling the HRT from the SRT. Using an UR with biomass retention, chain elongation was performed using a HRT of only 4 h, resulting in a maximum MCCA production rate of 57.4 g $L^{-1}d^{-1}$ using a synthetic ethanol and C2 feed supplemented with methanogenic inhibitors, yeast extract and CO_2 at neutral pH [105]. In addition, decreasing HRT reduces product toxicity by eliminating accumulation, e.g., by gradually reducing HRT from 20 to 2.5 days C6 production from cheese whey rate increased to ~5 gCOD $L^{-1}d^{-1}$ [100].

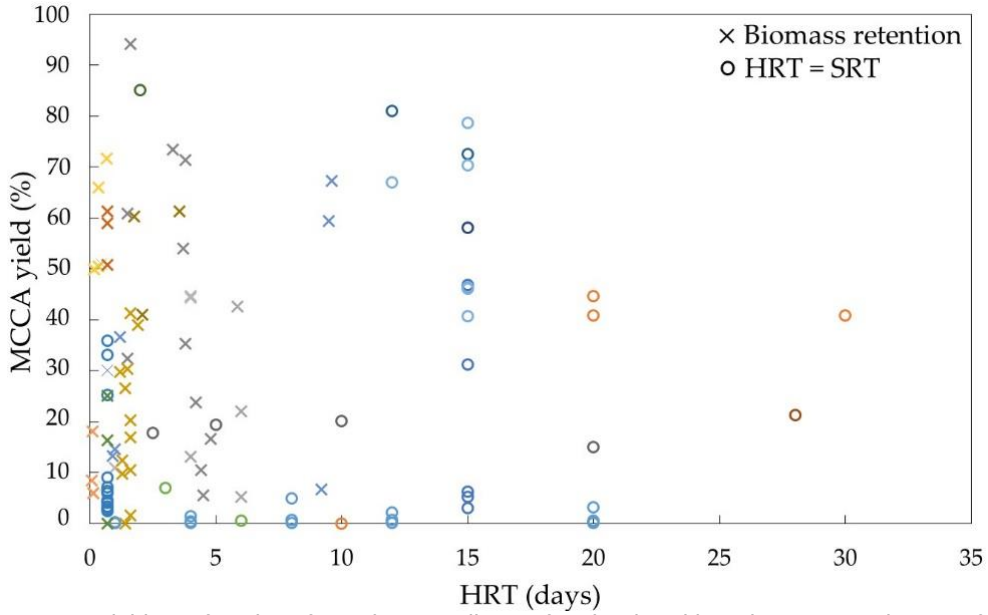


Figure 2-5 *MCCA yield as a function of HRT in 24 studies performing (semi-)continuous experiments of complex or synthetic feedstock. Colours represent experimental results reported within the same study. Estimated values were calculated as reported in Supplementary. Data collected from [23, 27, 36, 37, 72, 77, 88, 93-98, 100, 105, 106, 110, 111, 115, 123, 126-128, 170], the full data for the figure can be consulted in the following database [55].*

It is important to note that other operational factors come in to play alongside HRT. For instance, increasing HRT from 8 to 12 days and operating at 35 °C increased C6 concentrations in food waste fermentation from approx. 1.64 gCOD L^{-1} to 6.55 gCOD L^{-1}, and even more C6 (10.26 gCOD L^{-1}) was obtained at a HRT of 8 days by operating at 45 °C [125]. Therefore, the optimal HRT to stimulate chain elongation in complex feedstock fermentations will vary according to the type of system used, as it strongly depends on the reactor configuration, hydrolysis rate, sludge retention time, and other operational parameters such as pH and temperature.

2.1.10. Overcoming product toxicity by *in situ* extraction, biofilm formation or acclimation

Low MCCA concentrations in the fermentation broth result in poor product recovery and high cost of down-stream processing. MCCA concentration can be limited in MMC fermentation for three reasons: (i) the substrate is poor in electron donors or in easily biodegradable organics, and hence prevents *in situ* substrate accumulation to drive chain elongation; (ii) product accumulation lowers the thermodynamic favourability of chain elongation; and (iii) the antimicrobial properties of MCCA in their protonated state can result in product toxicity. To evaluate which cause is limiting MCCA production, Weimer et al. [40] measured residual substrate and product concentrations and calculated ΔG for C6 production. Incomplete substrate consumption in their MMC fermentation study enriched with *C. kluyveri* and ethanol-supplemented lignocellulosic feedstock still gave a negative

ΔG, thus suggesting product toxicity and/or limited incubation time had prevented further MCCA production [40]. Toxicity limits can be offset by employing *in situ* extraction, or biofilm formation and/or acclimation of the MMC.

In situ extraction methods have the advantage of continuously removing carboxylic acids from the fermentation broth, thereby alleviating product toxicity and thermodynamic constraints [130]. Various *in situ* extraction systems for carboxylic acids have been proposed for pure culture and MMC fermentation (Table 2-6). Electrochemical extraction has been applied to recover C2 to C6 from stillage fermentation, and has shown to simultaneously control pH and stimulate chain elongation by OH^- and H_2 production at the cathode [23]. However, for MMC fermentation of a complex substrate, a MCCA-selective extraction method is preferred to maintain low MCCA concentrations, whilst VFA remain in the fermentation broth as substrates for chain elongation. Pertraction has been used in various MMC studies as an *in situ* extraction method selective for MCCA. This is an in-line liquid-liquid, membrane-assisted extraction method driven by a pH-gradient, and is usually performed with mineral oil containing a phase transfer catalyst, e.g., TOPO, and an alkaline recovery phase [37, 77, 93, 96, 130]. Pertraction can be combined with membrane electrolysis to drive further separation and obtain a MCCA-rich oil [171]. Whilst the majority of *in situ* extraction studies report enhanced production rates and chain elongation, two studies did not report a significant improvement [62, 155]. The advantages of implementing *in situ* extraction systems must outweigh the increased complexity and cost in process operation.

Recently, different strategies to overcome product toxicity have been suggested. Allowing biofilms and microscale aggregates to develop improves interactions within a microbiome and tolerance to toxic compounds [172]. Addition of 2 g L^{-1} biochar to a UR fed with synthetic ethanol and C2 significantly improved MCCA production and reduced by-product formation [102]. In this case, microscopic observations revealed the community structure and the spatial distribution of microorganisms changed to dense microbial aggregates around the biochar; it was postulated this improved cell-cell interactions and energy efficiency via stabilising relationships between trophic partners, and increased tolerance to product toxicity [102]. Formation of microbial aggregates has been noted for bioreactors without providing a specific means of biofilm formation; granules were formed in a C3 and ethanol fed CSTR producing C7 as chain elongation product [170]. Therefore, reactor configuration and feedstock that allows microbial aggregates are expected to improve chain elongation, yet research on this is limited. In addition, recent research found the MCCA concentration in the fermentation broth influenced the microbial community structure. It has been suggested that elevated C6 and C8 concentrations lead to a more acclimatized and

resistant MMC [92, 166]. A microbiome adapted to operating at elevated C6 concentrations had a 10 times higher productivity in an environment with elevated C6 (33 gCOD L^{-1}) [160]. Further development of a resistant, highly productive MMC would allow accumulation of MCCA resulting in less complex extraction methods with reduced economic burden due to downstream processing.

Table 2-6 *Overview of the results of applying in situ extraction in MCCA fermentation experiments.*

***In Situ* Extraction and Fermentation Method**	**Results**	**Ref.**
Biphasic Extractive Fermentation		
M. elsdenii strain, sucrose substrate	58.5 gCOD L^{-1} C6 in solvent	[173]
Clostridium sp. BS-1, galactitol substrate	70.6 gCOD L^{-1} C6 in solvent	[174]
Anion Exchange Resin		
M. elsdenii strain, glucose substrate	Extraction doubled C6 to 24.3 gCOD L^{-1}	[175]
Integrated Cross-flow Nanofiltration		
MMC, pre-treated cellulosic feedstock	7× lower carboxylic acid concentration in fermenter, no significant yield improvement	[155]
Membrane electrolysis		
MMC, thin stillage feedstock	Lower need for caustic soda addition, C4-C6 from 46% to 70%, cathodic H_2 formation	[23]
Pertraction		
MMC, diluted yeast fermentation beer	MCCA-specific, 4× increase in C6 specificity to 32%, elongation to C8, extraction optimization	[37, 93, 130]
MMC, Synthetic substrate with C2 and ethanol	4× increase C8 productivity to 0.8 gCOD $L^{-1}d^{-1}$	[96]
MMC, Synthetic substrate with C4 and lactic acid	Productivity from 0.6 to 1 gCOD L^{-1} d^{-1} C6 by implementing extraction	[77]
C. kluyveri, synthetic substrate with C2 and ethanol	No significant difference in production rates	[62]
Pertraction and membrane electrolysis		
MMC, diluted fermentation beer	Lower need for caustic soda addition, MCCA recovery of 87%, obtained >90% MCCA oil	[171]

2.1.11. MMC fermentation scale up and integration within a bio-refinery context

Biological MCCA production is mostly in the experimental phase, but some scale-up has also been studied. For example, Hegner et al. [101] showed MCCA-producing MMC can be scaled up from 0.11 L serum bottles to 2.2 L bioreactors by maintaining similar reactor operation and without loss of performance or a change in microbial composition. Pilot scale projects are now being started. For instance, the MixAlco^{TM} process has been operated in four parallel 3.78 m^3 scale fed-batch fermenters processing chicken manure, urea and shredded paper to produce a mixture of carboxylate salts from processed fermentation effluent (containing approx. 6.8 gCOD L^{-1} C6) in an 11-month time period as precursors for jet fuel and gasoline [176]. The first start-ups and university spin-offs using MMC fermentation for production of MCCA and other bio-based standard chemicals as starting to appear, such as ChainCraft B.V. in the Netherlands [177].

In order to increase the potential of food waste as a feedstock for production of renewable chemicals, MCCA-producing MMC fermentation can be integrated within a bio-refinery. The term bio-refinery, defined as “the sustainable processing of biomass into a spectrum of marketable products and energy”, is inspired by traditional oil refineries where biomass replaces fossil fuels as feedstock for coproducing chemicals and power through various conversion technologies [178]. Process integration allows production of various compounds such as fuels, chemicals, solvents, biomaterials, food and feed ingredients, fibres and heat and power, thus increasing resilience and robustness against market price fluctuations while minimizing waste [179]. Using bio-waste as the renewable biomass feedstock, known as the 3rd generation bio-refinery concept, not only allows replacement of fossil fuel sources with a renewable alternative, but also stabilises waste streams with maximal use of resource, thus contributing to a circular economy [15]. Integration into a bio-refinery concept could include mechanical pre-treatment of waste streams to obtain pumpable mixtures, or pre-fermentation steps to obtain streams rich in lactic acid or ethanol. The combination of physical and biological processes for organic waste valorisation including MCCA will favour a myriad of product and energy goods that are market competitive (Table 2-7). Agler et al. gave an overview of bio-, thermo-, or electro-chemical post-processes, to convert carboxylic acids from fermentation into carbonyls, esters, alcohols or alkanes applicable as bulk fuels, solvents [26].

Table 2-7 *Processes and applications for carboxylic acid-rich (MCCA and VFA) fermentation effluent currently described in literature.*

Process/application	Product	Ref.
MixAlco process	Alcohol fuel	[28]
Secondary fermentation	Lipid/biodiesel	[180]
Secondary fermentation	Polyhydroxyalkanoates (PHA)	[147, 154, 181]
Microbial fuel cell feedstock	Electricity	[182]
Carbon source for bioremediation	Dechlorination of Chloroethenes	[183]
Extraction and Kolbe electrolysis	Liquid alkane fuels, i.e., C10-C20 hydrocarbons	[14, 27, 95]

2.1.12. Conclusions

MCCA, such as caproic and caprylic acid, are compounds of interest due to their broad range of potential applications. In contrast to chemical or single-culture biotechnological processes, using the consorted action of MMC allows to produce MCCA from complex organic feedstocks, such as food waste, in open, non-sterile systems via the natural process of chain elongation. However, the yields, concentrations and selectivity of this process must be improved in order to increase its viability. Therefore, we have summarised the current knowledge on the underlying mechanism of chain elongation by MMC, discussed the current state of the art on the use of complex organic feedstock and reviewed key operational parameters, and their interactions.

Some of the key findings lie with the fact that with complex substrates and microbial cultures, there must be a greater emphasis on managing competing reactions and positively selecting for chain elongation microbiomes. Since the microbial diversity of MMC ecosystems has been shown to be distinct from pure cultures and clean substrates, existing thermodynamic and kinetic models should be expanded to include complex feedstock and mixed cultures. Advances in microbial culture analysis, such as improved implementation of various “-omics” methods on complex samples, will boost current understanding of MMC fermentation.

Most common complex feedstocks trialled so far include residues from the bio-ethanol and dairy industries, different types of cellulosic wastes, syngas fermentation effluent and different types of organic food waste. These type of feedstocks have resulted in maximum production rates up to 8.02 gCOD $L^{-1}d^{-1}$. Supplementation of complex waste-derived feedstock with chain elongation substrates such as ethanol increased production rates,

with maxima reported up to 62.8 gCOD $L^{-1}d^{-1}$. However, the negative environmental effects from chemical addition have also been reported. The use of synthetic substrates allowed production rates up to 115.2 gCOD $L^{-1}d^{-1}$.

Through an extensive review of the literature, including studies targeting MCCA or reporting MCCA as by-products, various key operational parameters were identified and discussed to highlight the research gaps. Mesophilic temperatures are so far a preferred choice for chain elongation, yet there is little justification for this. The preferred operational pH seems to lie in a slight acidic range from pH 5 to 7, in order to limit the activity of methanogens. The relationship between organic loading rate (OLR) and MCCA production rates showed a positive correlation to some extent, however this is complicated by the degree of biodegradability of the feedstock. Linked to the organic load is the substrate-inoculum ratio (F/M) at the start-up of the process which favours the accumulation of intermediate compounds instead of methane production when F/M > 5. In addition, whilst increased OLR tends to improve chain elongation, this must be coupled with sufficiently long residence times and biomass retention. OLR and retention times will have to be optimized depending on whether the reactor design has included mechanisms for biomass retention, and the biodegradability of the feedstock.

The literature study revealed very little information is available on some specific operational parameters that have been studied for other MMC applications. For example, in similar MMC fermentation processes a minimal alkalinity was beneficial to stabilise the process and reduce the need for pH controlling agents. However, the buffer capacity required to stimulate chain elongation has not been thoroughly investigated. The partial pressures of CO_2 and H_2 in the reactor headspace have been identified to influence chain elongation, however production of these gases during fermentation, and their accumulation in reactor headspace is rarely considered. In order to circumvent the antimicrobial limitations imposed by MCCA on the microbiome, *in situ* extraction is often proposed, but the alternative strategies which promote the development of biofilm or granule formation, and MMC adaptation, are worthy of further research. Finally, the development of down-stream processing methods, and integration within a bio-refinery context, are crucial issues to transform MCCA production from organic waste streams into a competitive waste valorisation technology that will contribute to the development of a circular economy.

Acknowledgements and funding

This research was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement H2020-MSCA-CO-FUND,

No 665992, and Wessex Water and GENeco. The Centre for Sustainable Chemical Technologies is supported by EPSRC under grant EP/L016354/1.

References

[1] Eurostat the statistical office of the European Union, "Recycling of biowaste (cei_wm030)," ed. Luxembourg: Eurostat the statistical office of the European Union, 2018.

[2] L. Appels *et al.*, "Anaerobic digestion in global bio-energy production: Potential and research challenges," *Renewable and Sustainable Energy Reviews,* vol. 15, no. 9, pp. 4295-4301, 2011.

[3] S. Vakalis, A. Sotiropoulos, K. Moustakas, D. Malamis, K. Vekkos, and M. Baratieri, "Thermochemical valorization and characterization of household biowaste," *J Environ Manage,* vol. 203, no. Pt 2, pp. 648-654, Dec 1 2017.

[4] A. Molino, V. Larocca, S. Chianese, and D. Musmarra, "Biofuels Production by Biomass Gasification: A Review," *Energies,* vol. 11, no. 4, 2018.

[5] A. Kumar and S. R. Samadder, "A review on technological options of waste to energy for effective management of municipal solid waste," *Waste Manag,* vol. 69, pp. 407-422, Nov 2017.

[6] T. P. Pham, R. Kaushik, G. K. Parshetti, R. Mahmood, and R. Balasubramanian, "Food waste-to-energy conversion technologies: current status and future directions," *Waste Manag,* vol. 38, pp. 399-408, Apr 2015.

[7] A. Naresh Kumar and S. Venkata Mohan, "Acidogenic valorization of vegetable waste for short chain carboxylic acids and biohydrogen production: Influence of pretreatment and pH," *Journal of Cleaner Production,* vol. 203 pp. 1055-1066, 2018.

[8] P. K. Sadh, S. Kumar, P. Chawla, and J. S. Duhan, "Fermentation: A Boon for Production of Bioactive Compounds by Processing of Food Industries Wastes (By-Products)," *Molecules,* vol. 23, no. 10, Oct 8 2018.

[9] M. Coma *et al.*, "Organic waste as a sustainable feedstock for platform chemicals," *Faraday Discuss,* vol. 202, pp. 175-195, Sep 21 2017.

[10] C. O. Tuck, E. Perez, I. T. Horvath, R. a. Sheldon, and M. Poliakoff, "Valorization of Biomass: Deriving more value from waste," *Science,* review vol. 337, pp. 695-699, 2012.

[11] L. T. Angenent and B. A. Wrenn, "Optimizing Mixed-Culture Bioprocessing To Convert Wastes into Bioenergy," in *Bioenergy*, J. Wall, C. Harwood, and A. Demain, Eds. Washington, DC, USA: American Society of Microbiology, 2008, pp. 179-194.

[12] J. P. H. van Wyk, "Biotechnology and the utilization of biowaste as a resource for bioproduct development," *Trends in biotechnology,* vol. 19, no. 5, pp. 172-177, 2001.

[13] F. Fava *et al.*, "Biowaste biorefinery in Europe: opportunities and research & development needs," *N Biotechnol,* vol. 32, no. 1, pp. 100-8, Jan 25 2015.

[14] P. F. Levy, J. E. Sanderson, R. G. Kispert, and D. L. Wise, "Biorefining of biomass to liquid fuels and organic chemicals," *Enzyme Microb. Technol.,* vol. 3, no. 207-215, 1981.

[15] S. Venkata Mohan *et al.*, "Waste biorefinery models towards sustainable circular bioeconomy: Critical review and future perspectives," *Bioresour Technol,* vol. 215, pp. 2-12, Sep 2016.

[16] D. Meyer-Kohlstock, G. Hadrich, W. Bidlingmaier, and E. Kraft, "The value of composting in Germany--economy, ecology, and legislation," *Waste Manag,* vol. 33, no. 3, pp. 536-9, Mar 2013.

[17] H. Fisgativa, A. Tremier, S. Le Roux, C. Bureau, and P. Dabert, "Understanding the anaerobic biodegradability of food waste: Relationship between the typological,

biochemical and microbial characteristics," *J Environ Manage,* vol. 188, pp. 95-107, Mar 1 2017.
[18] J. De Vrieze, M. Coma, M. Debeuckelaere, P. Van der Meeren, and K. Rabaey, "High salinity in molasses wastewaters shifts anaerobic digestion to carboxylate production," *Water Res,* vol. 98, pp. 293-301, Jul 1 2016.
[19] D. J. Anneken, S. Both, R. Christoph, G. Fieg, U. Steinberner, and A. Westfechtel, "Fatty Acids," in *Ullmann's Encyclopedia of Industrial Chemistry*, vol. 14Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 2006, pp. 73-116.
[20] A. Perimenis, T. Nicolay, M. Leclercq, and P. A. Gerin, "Comparison of the acidogenic and methanogenic potential of agroindustrial residues," *Waste Manag,* vol. 72, pp. 178-185, Feb 2018.
[21] S. Dahiya, A. N. Kumar, J. Shanthi Sravan, S. Chatterjee, O. Sarkar, and S. V. Mohan, "Food waste biorefinery: Sustainable strategy for circular bioeconomy," *Bioresour Technol,* vol. 248, no. Pt A, pp. 2-12, Jan 2018.
[22] M.-P. Zacharof and R. W. Lovitt, "Complex Effluent Streams as a Potential Source of Volatile Fatty Acids," *Waste and Biomass Valorization,* vol. 4, no. 3, pp. 557-581, 2013.
[23] S. J. Andersen *et al.*, "Electrolytic extraction drives volatile fatty acid chain elongation through lactic acid and replaces chemical pH control in thin stillage fermentation," *Biotechnol Biofuels,* vol. 8, p. 221, 2015.
[24] F. Van Immerseel *et al.*, "The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy," *Avian Pathol,* vol. 35, no. 3, pp. 182-8, Jun 2006.
[25] F. Solis de los Santos *et al.*, "The natural feed additive caprylic acid decreases Campylobacter jejuni colonization in market-aged broiler chickens," *Poult Sci,* vol. 88, no. 1, pp. 61-4, Jan 2009.
[26] M. T. Agler, B. A. Wrenn, S. H. Zinder, and L. T. Angenent, "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform," *Trends Biotechnol,* vol. 29, no. 2, pp. 70-8, Feb 2011.
[27] C. Urban *et al.*, "Production of drop-in fuels from biomass at high selectivity by combined microbial and electrochemical conversion," *Energy & Environmental Science,* vol. 10, no. 10, pp. 2231-2244, 2017.
[28] M. T. Holtzapple *et al.*, "Biomass Conversion to Mixed Alcohol Fuels using the MixAlco process," *Applied Biochemistry and Biotechnology,* vol. 77-79, pp. 609-631, 1999.
[29] C. M. Spirito, H. Richter, K. Rabaey, A. J. Stams, and L. T. Angenent, "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr Opin Biotechnol,* vol. 27, pp. 115-22, Jun 2014.
[30] P. J. Weimer and R. A. Kohn, "Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside?," *Appl Microbiol Biotechnol,* vol. 100, no. 8, pp. 3389-98, Apr 2016.
[31] J. Whipps, K. Lewis, and R. Cooke, "Chapter 9. Mycoparasitism and plant disease control," in *Fungi in Biological Control Systems*, M. N. Burge, Ed.: Manchester University Press, 1988, pp. 161-187.
[32] A. Lahel *et al.*, "Effect of process parameters on the bioremediation of diesel contaminated soil by mixed microbial consortia," *International Biodeterioration & Biodegradation,* vol. 113, pp. 375-385, 2016.
[33] M. Jariyal, V. Jindal, K. Mandal, V. K. Gupta, and B. Singh, "Bioremediation of organophosphorus pesticide phorate in soil by microbial consortia," *Ecotoxicology and Environmental Safety,* vol. 159, pp. 310-316, 2018.
[34] A. Bechamp, "Lettre de M.A. Bechamp a M. Dumas," vol. 6, ed. Paris, France: Annales de chimie et de physique, Gallica Bibliothèque de Météo-France, 1867.
[35] H. A. Barker, "The production of caproic and butyric acids by the methane fermentation of ethyl alcohol," *Laboratory of Microbiology, Technical University, Delft, Holland.,* 1937.

[36] M. T. Agler *et al.*, "Shaping reactor microbiomes to produce the fuel precursor n-butyrate from pretreated cellulosic hydrolysates," *Environ Sci Technol,* vol. 46, no. 18, pp. 10229-38, Sep 18 2012.
[37] S. Ge, J. G. Usack, C. M. Spirito, and L. T. Angenent, "Long-Term n-Caproic Acid Production from Yeast-Fermentation Beer in an Anaerobic Bioreactor with Continuous Product Extraction," *Environ Sci Technol,* vol. 49, no. 13, pp. 8012-21, Jul 7 2015.
[38] S. Widder *et al.*, "Challenges in microbial ecology: building predictive understanding of community function and dynamics," *ISME J,* vol. 10, no. 11, pp. 2557-2568, Nov 2016.
[39] W. R. Kenealy, Y. Cao, and P. J. Weimer, "Production of caproic acid by cocultures of ruminal cellulolytic bacteria and Clostridium kluyveri grown on cellulose and ethanol," *appl Microbiol Biotechnol,* vol. 44, pp. 507-513, 1995.
[40] P. J. Weimer, M. Nerdahl, and D. J. Brandl, "Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri," *Bioresour Technol,* vol. 175, pp. 97-101, Jan 2015.
[41] L. Leng, P. Yang, Y. Mao, Z. Wu, T. Zhang, and P. H. Lee, "Thermodynamic and physiological study of caproate and 1,3-propanediol co-production through glycerol fermentation and fatty acids chain elongation," *Water Res,* vol. 114, pp. 200-209, May 1 2017.
[42] M. Diender, A. J. Stams, and D. Z. Sousa, "Production of medium-chain fatty acids and higher alcohols by a synthetic co-culture grown on carbon monoxide or syngas," *Biotechnol Biofuels,* vol. 9, p. 82, 2016.
[43] P. He, W. Han, L. Shao, and F. Lu, "One-step production of C6-C8 carboxylates by mixed culture solely grown on CO," *Biotechnol Biofuels,* vol. 11, p. 4, 2018.
[44] M. C. A. A. Van Eerten-Jansen *et al.*, "Bioelectrochemical Production of Caproate and Caprylate from Acetate by Mixed Cultures," *ACS Sustainable Chemistry & Engineering,* vol. 1, no. 5, pp. 513-518, 2013.
[45] I. Vassilev *et al.*, "Microbial Electrosynthesis of Isobutyric, Butyric, Caproic Acids, and Corresponding Alcohols from Carbon Dioxide," *ACS Sustainable Chemistry & Engineering,* vol. 6, no. 7, pp. 8485-8493, 2018.
[46] C. W. Marshall, E. V. LaBelle, and H. D. May, "Production of fuels and chemicals from waste by microbiomes," *Curr Opin Biotechnol,* vol. 24, no. 3, pp. 391-7, Jun 2013.
[47] D. Arslan *et al.*, "Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations," *Critical Reviews in Environmental Science and Technology,* vol. 46, no. 6, pp. 592-634, 2016.
[48] E. A. F. Vasconcelos, R. C. Leitão, and S. T. Santaella, "Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors," *BioEnergy Research,* vol. 9, no. 4, pp. 1260-1271, 2016.
[49] M. V. Reddy, A. ElMekawy, and D. Pant, "Bioelectrochemical synthesis of caproate through chain elongation as a complementary technology to anaerobic digestion," *Biofuels, Bioproducts and Biorefining,* vol. 12, no. 6, pp. 966-977, 2018.
[50] R. Kleerebezem, B. Joosse, R. Rozendal, and M. C. M. Van Loosdrecht, "Anaerobic digestion without biogas?," *Reviews in Environmental Science and Bio/Technology,* vol. 14, no. 4, pp. 787-801, 2015.
[51] J.-R. Bastidas-Oyanedel, F. Bonk, M. H. Thomsen, and J. E. Schmidt, "Dark fermentation biorefinery in the present and future (bio)chemical industry," *Reviews in Environmental Science and Bio/Technology,* vol. 14, no. 3, pp. 473-498, 2015.
[52] C. M. Braguglia, A. Gallipoli, A. Gianico, and P. Pagliaccia, "Anaerobic bioconversion of food waste into energy: A critical review," *Bioresour Technol,* vol. 248, no. Pt A, pp. 37-56, Jan 2018.
[53] G. Capson-Tojo, M. Rouez, M. Crest, J.-P. Steyer, J.-P. Delgenès, and R. Escudié, "Food waste valorization via anaerobic processes: a review," *Reviews in Environmental Science and Bio/Technology,* vol. 15, no. 3, pp. 499-547, 2016.

[54] B. Sen, J. Aravind, P. Kanmani, and C.-H. Lay, "State of the art and future concept of food waste fermentation to bioenergy," *Renewable and Sustainable Energy Reviews,* vol. 53, pp. 547-557, 2016.
[55] V. De Groof, M. Coma, T. Arnot, D. Leak, and A. Lanham, "Dataset on experimental data available in the literature on "Medium chain carboxylic acids from complex organic feedstock by mixed culture fermentation"," ed. University of Bath Research Data Archive, 2019.
[56] H. Seedorf *et al.*, "The genome of Clostridium kluyveri, a strict anaerobe with unique metabolic features," *PNAS* vol. 105, no. 6, pp. 21128-2133, 2008.
[57] W. R. Kenealy and D. M. Waselefsky, "Studies on the substrate range of Clostridium kluyveri; the use of propanol and succinate " *Archives of Microbiology,* vol. 141, pp. 187-194, 1985.
[58] L. T. Angenent *et al.*, "Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals," *Environ Sci Technol,* vol. 50, no. 6, pp. 2796-810, Mar 15 2016.
[59] Y. Yin, Y. Zhang, D. B. Karakashev, J. Wang, and I. Angelidaki, "Biological caproate production by Clostridium kluyveri from ethanol and acetate as carbon sources," *Bioresour Technol,* vol. 241, pp. 638-644, Oct 2017.
[60] P. J. Weimer and D. M. Stevenson, "Isolation, characterization, and quantification of Clostridium kluyveri from the bovine rumen," *Appl Microbiol Biotechnol,* vol. 94, no. 2, pp. 461-6, Apr 2012.
[61] R. K. Thauer, K. Jungermann, H. Henninger, J. Wenning, and K. Decker, "The energy metabolism of Clostridium kluyveri," *European Journal of Biochemistry,* vol. 4, pp. 173-180, 1968.
[62] S. Gildemyn, B. Molitor, J. G. Usack, M. Nguyen, K. Rabaey, and L. T. Angenent, "Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation," *Biotechnol Biofuels,* vol. 10, p. 83, 2017.
[63] M. Marounek, K. Fliegrova, and S. Bartos, "Metabolism and Some Characteristics of Ruminal Strains of Megasphaera elsdenii," *Applied and environmental microbiology,* vol. 55, no. 6, pp. 1570-1573, 1989.
[64] Y. Tao *et al.*, "Complete genome sequence of Ruminococcaceae bacterium CPB6: A newly isolated culture for efficient n-caproic acid production from lactate," *J Biotechnol,* vol. 259, pp. 91-94, Oct 10 2017.
[65] S. G. Kim *et al.*, "Optimization of hexanoic acid production in recombinant Escherichia coli by precise flux rebalancing," *Bioresource Technology,* vol. 247, pp. 1253-1257, 2018/01/01/ 2018.
[66] Y. Cheon *et al.*, "A biosynthetic pathway for hexanoic acid production in Kluyveromyces marxianus," *Journal of Biotechnology,* vol. 182-183, pp. 30-36, 2014/07/20/ 2014.
[67] S. Sarria, N. S. Kruyer, and P. Peralta-Yahya, "Microbial synthesis of medium-chain chemicals from renewables," *Nat Biotechnol,* vol. 35, no. 12, pp. 1158-1166, Dec 2017.
[68] H. Su, J. Lin, and F. Tan, "Progress and perspective of biosynthetic platform for higher-order biofuels," *Renewable and Sustainable Energy Reviews,* vol. 80, pp. 801-826, 2017.
[69] W. d. A. Cavalcante, R. C. Leitão, T. A. Gehring, L. T. Angenent, and S. T. Santaella, "Anaerobic fermentation for n-caproic acid production: A review," *Process Biochemistry,* vol. 54, pp. 106-119, 2017.
[70] K. J. J. Steinbusch, H. V. M. Hamelers, C. M. Plugge, and C. J. N. Buisman, "Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass," *Energy Environ. Sci.,* vol. 4, no. 1, pp. 216-224, 2011.
[71] R. Kleerebezem and M. C. van Loosdrecht, "Mixed culture biotechnology for bioenergy production," *Curr Opin Biotechnol,* vol. 18, no. 3, pp. 207-12, Jun 2007.

[72] T. I. Grootscholten, K. J. Steinbusch, H. V. Hamelers, and C. J. Buisman, "Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production," *Bioresour Technol,* vol. 135, pp. 440-5, May 2013.
[73] C. M. Spirito, A. M. Marzilli, and L. T. Angenent, "Higher Substrate Ratios of Ethanol to Acetate Steered Chain Elongation toward n-Caprylate in a Bioreactor with Product Extraction," *Environ Sci Technol,* vol. 52, no. 22, pp. 13438-13447, Nov 20 2018.
[74] T. I. Grootscholten, K. J. Steinbusch, H. V. Hamelers, and C. J. Buisman, "High rate heptanoate production from propionate and ethanol using chain elongation," *Bioresour Technol,* vol. 136, pp. 715-8, May 2013.
[75] M. Coma, R. Vilchez-Vargas, H. Roume, R. Jauregui, D. H. Pieper, and K. Rabaey, "Product Diversity Linked to Substrate Usage in Chain Elongation by Mixed-Culture Fermentation," *Environ Sci Technol,* vol. 50, no. 12, pp. 6467-76, Jun 21 2016.
[76] W. S. Chen, Y. Ye, K. J. J. Steinbusch, D. P. B. T. B. Strik, and C. J. N. Buisman, "Methanol as an alternative electron donor in chain elongation for butyrate and caproate formation," *Biomass and Bioenergy,* vol. 93, pp. 201-208, 2016.
[77] L. A. Kucek, M. Nguyen, and L. T. Angenent, "Conversion of L-lactate into n-caproate by a continuously fed reactor microbiome," *Water Res,* vol. 93, pp. 163-171, Apr 15 2016.
[78] B. E. Jackson and M. J. McInerney, "Anaerobic microbial metabolism can proceed close to thermodynamic limits," *Nature,* vol. 415, pp. 454-456, 2002.
[79] R. González-Cabaleiro, J. M. Lema, J. Rodríguez, and R. Kleerebezem, "Linking thermodynamics and kinetics to assess pathway reversibility in anaerobic bioprocesses," *Energy & Environmental Science,* vol. 6, no. 12, 2013.
[80] R. Kleerebezem and M. C. M. Van Loosdrecht, "A Generalized Method for Thermodynamic State Analysis of Environmental Systems," *Critical Reviews in Environmental Science and Technology,* vol. 40, no. 1, pp. 1-54, 2010.
[81] J. Rodriguez, R. Kleerebezem, J. M. Lema, and M. C. van Loosdrecht, "Modeling product formation in anaerobic mixed culture fermentations," *Biotechnol Bioeng,* vol. 93, no. 3, pp. 592-606, Feb 20 2006.
[82] K. W. Hanselmann, "Microbial energetics applied to waste repositories," *Experientia,* review vol. 47, pp. 645-687, 1991.
[83] M. V. Reddy, S. V. Mohan, and Y. C. Chang, "Sustainable production of medium chain fatty acids (MCFA) with an enriched mixed bacterial culture: microbial characterization using molecular methods," *Sustainable Energy & Fuels,* vol. 2, no. 2, pp. 372-380, 2018.
[84] Y. Chen, J. J. Cheng, and K. S. Creamer, "Inhibition of anaerobic digestion process: a review," *Bioresour Technol,* vol. 99, no. 10, pp. 4044-64, Jul 2008.
[85] P. Candry *et al.*, "A novel high-throughput method for kinetic characterisation of anaerobic bioproduction strains, applied to Clostridium kluyveri," *Sci Rep,* vol. 8, no. 1, p. 9724, Jun 27 2018.
[86] R. Gonzalez-Cabaleiro, J. M. Lema, and J. Rodriguez, "Metabolic energy-based modelling explains product yielding in anaerobic mixed culture fermentations," *PLoS One,* vol. 10, no. 5, p. e0126739, 2015.
[87] R. Prabhu, E. Altman, and M. A. Eiteman, "Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions," *Appl Environ Microbiol,* vol. 78, no. 24, pp. 8564-70, Dec 2012.
[88] X. Zhu *et al.*, "The synthesis of n-caproate from lactate: a new efficient process for medium-chain carboxylates production," *Sci Rep,* vol. 5, p. 14360, Sep 25 2015.
[89] H. Sträuber, M. Schröder, and S. Sabine Kleinsteuber, "Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process," *Energy, Sustainability and Society,* vol. 2, no. 13, 2012.
[90] M. V. Reddy, S. Hayashi, D. Choi, H. Cho, and Y. C. Chang, "Short chain and medium chain fatty acids production using food waste under non-augmented and bio-augmented conditions," *Journal of Cleaner Production,* vol. 176, pp. 645-653, 2018.

[91] C. O. Nzeteu, A. C. Trego, F. Abram, and V. O'Flaherty, "Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system," *Biotechnol Biofuels,* vol. 11, p. 108, 2018.
[92] S. J. Andersen *et al.*, "A Clostridium Group IV Species Dominates and Suppresses a Mixed Culture Fermentation by Tolerance to Medium Chain Fatty Acids Products," *Front Bioeng Biotechnol,* vol. 5, p. 8, 2017.
[93] M. T. Agler, C. M. Spirito, J. G. Usack, J. J. Werner, and L. T. Angenent, "Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates," *Energy & Environmental Science,* vol. 5, no. 8, 2012.
[94] J. Xu, J. Hao, J. J. L. Guzman, C. M. Spirito, L. A. Harroff, and L. T. Angenent, "Temperature-Phased Conversion of Acid Whey Waste Into Medium-Chain Carboxylic Acids via Lactic Acid: No External e-Donor," *Joule,* vol. 2, no. 2, pp. 280-295, 2018.
[95] W. C. Khor, S. Andersen, H. Vervaeren, and K. Rabaey, "Electricity-assisted production of caproic acid from grass," *Biotechnol Biofuels,* vol. 10, p. 180, 2017.
[96] L. A. Kucek, C. M. Spirito, and L. T. Angenent, "High n-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation," *Energy & Environmental Science,* vol. 9, no. 11, pp. 3482-3494, 2016.
[97] L. A. Kucek, J. Xu, M. Nguyen, and L. T. Angenent, "Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction," *Front Microbiol,* vol. 7, p. 1892, 2016.
[98] M. Roghair *et al.*, "Controlling Ethanol Use in Chain Elongation by CO2 Loading Rate," *Environ Sci Technol,* vol. 52, no. 3, pp. 1496-1505, Feb 6 2018.
[99] M. J. Scarborough, G. Lynch, M. Dickson, M. McGee, T. J. Donohue, and D. R. Noguera, "Increasing the economic value of lignocellulosic stillage through medium-chain fatty acid production," *Biotechnol Biofuels,* vol. 11, p. 200, 2018.
[100] A. Duber *et al.*, "Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey," *Green Chemistry,* vol. 20, no. 16, pp. 3790-3803, 2018.
[101] R. Hegner, C. Koch, V. Riechert, and F. Harnisch, "Microbiome-based carboxylic acids production: from serum bottles to bioreactors," *RSC Advances,* vol. 7, no. 25, pp. 15362-15371, 2017.
[102] Y. Liu, P. He, L. Shao, H. Zhang, and F. Lu, "Significant enhancement by biochar of caproate production via chain elongation," *Water Res,* vol. 119, pp. 150-159, Aug 1 2017.
[103] M. J. Scarborough, C. E. Lawson, J. J. Hamilton, T. J. Donohue, and D. R. Noguera, "Metatranscriptomic and Thermodynamic Insights into Medium-Chain Fatty Acid Production Using an Anaerobic Microbiome," *mSystems,* 2018.
[104] W. Han, P. He, L. Shao, and F. Lü, "Metabolic Interactions of a Chain Elongation Microbiome," *Applied and Environmental Microbiology,* 10.1128/AEM.01614-18 vol. 84, no. 22, 2018.
[105] T. I. Grootscholten, K. J. Steinbusch, H. V. Hamelers, and C. J. Buisman, "Improving medium chain fatty acid productivity using chain elongation by reducing the hydraulic retention time in an upflow anaerobic filter," *Bioresour Technol,* vol. 136, pp. 735-8, May 2013.
[106] T. I. M. Grootscholten, D. P. B. T. B. Strik, K. J. J. Steinbusch, C. J. N. Buisman, and H. V. M. Hamelers, "Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol," *Applied Energy,* vol. 116, pp. 223-229, 2014.
[107] S. Lonkar, Z. Fu, and M. Holtzapple, "Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate," *Biotechnol Bioeng,* vol. 113, no. 12, pp. 2597-2604, Dec 2016.

[108] T. I. M. Grootscholten, F. Kinsky dal Borgo, H. V. M. Hamelers, and C. J. N. Buisman, "Promoting chain elongation in mixed culture acidification reactors by addition of ethanol," *Biomass and Bioenergy,* vol. 48, pp. 10-16, 2013.
[109] W. S. Chen, D. Strik, C. J. N. Buisman, and C. Kroeze, "Production of Caproic Acid from Mixed Organic Waste: An Environmental Life Cycle Perspective," *Environ Sci Technol,* vol. 51, no. 12, pp. 7159-7168, Jun 20 2017.
[110] D. Vasudevan, H. Richter, and L. T. Angenent, "Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes," *Bioresour Technol,* vol. 151, pp. 378-82, Jan 2014.
[111] J. M. B. Domingos *et al.*, "Effect of Operational Parameters in the Continuous Anaerobic Fermentation of Cheese Whey on Titers, Yields, Productivities, and Microbial Community Structures," *ACS Sustainable Chemistry & Engineering,* vol. 5, no. 2, pp. 1400-1407, 2016.
[112] J. Tang, X. C. Wang, Y. Hu, Y. Zhang, and Y. Li, "Effect of pH on lactic acid production from acidogenic fermentation of food waste with different types of inocula," *Bioresour Technol,* vol. 224, pp. 544-552, Jan 2017.
[113] K. Sakai *et al.*, "Selective proliferation of lactic acid bacteria and accumulation of lactic acid during open fermentation of kitchen refuse with intermittent pH adjustments," *Food Science and Technology Resource,* vol. 6, no. 2, pp. 140-145, 2000.
[114] F. Bonk, J. R. Bastidas-Oyanedel, A. F. Yousef, and J. E. Schmidt, "Exploring the selective lactic acid production from food waste in uncontrolled pH mixed culture fermentations using different reactor configurations," *Bioresour Technol,* vol. 238, pp. 416-424, Aug 2017.
[115] Y. Wang, Y. Mu, and H. Yu, "Comparative performance of two upflow anaerobic biohydrogen-producing reactors seeded with different sludges," *International Journal of Hydrogen Energy,* vol. 32, no. 8, pp. 1086-1094, 2007.
[116] X. Gómez, M. J. Cuetos, J. I. Prieto, and A. Morán, "Bio-hydrogen production from waste fermentation: Mixing and static conditions," *Renewable Energy,* vol. 34, no. 4, pp. 970-975, 2009.
[117] D. Arslan, K. J. Steinbusch, L. Diels, H. De Wever, C. J. Buisman, and H. V. Hamelers, "Effect of hydrogen and carbon dioxide on carboxylic acids patterns in mixed culture fermentation," *Bioresour Technol,* vol. 118, pp. 227-34, Aug 2012.
[118] L. Chatellard, E. Trably, and H. Carrere, "The type of carbohydrates specifically selects microbial community structures and fermentation patterns," *Bioresour Technol,* vol. 221, pp. 541-549, Dec 2016.
[119] D. Shen *et al.*, "Acidogenic fermentation characteristics of different types of protein-rich substrates in food waste to produce volatile fatty acids," *Bioresour Technol,* vol. 227, pp. 125-132, Mar 2017.
[120] O. P. Karthikeyan, S. Mehariya, and J. W. C. Wong, "Biorefining of food waste for fuel and energy," presented at the 4th International Conference on Energy and Environment Research, ICEER, Porto, Portugal, 2017. Review.
[121] J. Kannengiesser, K. Sakaguchi-Soder, T. Mrukwia, J. Jager, and L. Schebek, "Extraction of medium chain fatty acids from organic municipal waste and subsequent production of bio-based fuels," *Waste Manag,* vol. 47, no. Pt A, pp. 78-83, Jan 2016.
[122] F. C. Silva, L. S. Serafim, H. Nadais, L. Arroja, and I. Capelaa, "Acidogenic fermentation towards valorisation of organic waste streams into volatile fatty acids," *Chem. Biochem. Eng. Q.,* vol. 27, no. 4, pp. 467-476, 2013.
[123] E. Jankowska, A. Duber, J. Chwialkowska, M. Stodolny, and P. Oleskowicz-Popiel, "Conversion of organic waste into volatile fatty acids – The influence of process operating parameters," *Chemical Engineering Journal,* vol. 345, pp. 395-403, 2018.
[124] S. B. Domke, C. Aiello-Mazzarri, and M. T. Holtzapple, "Mixed acid fermentation of paper fines and industrial biosludge," *Bioresource Technology,* vol. 91, no. 1, pp. 41-51, 2004.

[125] S. J. Lim, B. J. Kim, C. M. Jeong, J. D. Choi, Y. H. Ahn, and H. N. Chang, "Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor," *Bioresour Technol,* vol. 99, no. 16, pp. 7866-74, Nov 2008.
[126] I. O. Bolaji and D. Dionisi, "Acidogenic fermentation of vegetable and salad waste for chemicals production: Effect of pH buffer and retention time," *Journal of Environmental Chemical Engineering,* vol. 5, no. 6, pp. 5933-5943, 2017.
[127] H. B. Ding, G. Y. Tan, and J. Y. Wang, "Caproate formation in mixed-culture fermentative hydrogen production," *Bioresour Technol,* vol. 101, no. 24, pp. 9550-9, Dec 2010.
[128] M. Roghair, Y. Liu, D. Strik, R. A. Weusthuis, M. E. Bruins, and C. J. N. Buisman, "Development of an Effective Chain Elongation Process From Acidified Food Waste and Ethanol Into n-Caproate," *Front Bioeng Biotechnol,* vol. 6, p. 50, 2018.
[129] E. B. Hollister *et al.*, "Structure and dynamics of the microbial communities underlying the carboxylate platform for biofuel production," *Appl Microbiol Biotechnol,* vol. 88, no. 1, pp. 389-99, Sep 2010.
[130] M. T. Agler, C. M. Spirito, J. G. Usack, J. J. Werner, and L. T. Angenent, "Development of a highly specific and productive process for n-caproic acid production: applying lessons from methanogenic microbiomes," *Water Sci Technol,* vol. 69, no. 1, pp. 62-8, 2014.
[131] E. B. Hollister *et al.*, "Mesophilic and thermophilic conditions select for unique but highly parallel microbial communities to perform carboxylate platform biomass conversion," *PLoS One,* vol. 7, no. 6, p. e39689, 2012.
[132] N. Tomlinson and H. A. Barker, "Carbon dioxide and acetate utilization by clostridium kluyveri: I. influence of nutritional conditions on utilization patterns," *J. Biol. Chem.,* vol. 209, pp. 585-595, 1953.
[133] S. Liang and C. Wan, "Carboxylic acid production from brewer's spent grain via mixed culture fermentation," *Bioresour Technol,* vol. 182, pp. 179-183, Apr 2015.
[134] I. Angelidaki and W. Sanders, "Assessment of the anaerobic biodegradability of macropollutants," *Reviews in Environmental Science and Bio/Technology* vol. 3, pp. 117-129, 2004.
[135] E. D'Addario, R. Pappa, B. Pietrangeli, and M. Valdiserri, "The acidogenic digestion of the organic fraction of municipal solid waste for the production of liquid fuels," *Water Sci Technol,* vol. 27, no. 2, pp. 183-192, 1993.
[136] Y. Maspolim, Y. Zhou, C. Guo, K. Xiao, and W. J. Ng, "The effect of pH on solubilization of organic matter and microbial community structures in sludge fermentation," *Bioresour Technol,* vol. 190, pp. 289-98, Aug 2015.
[137] J. Garcia-Aguirre, E. Aymerich, J. González-Mtnez. de Goñi, and M. Esteban-Gutiérrez, "Selective VFA production potential from organic waste streams: Assessing temperature and pH influence," *Bioresource Technology,* vol. 244, pp. 1081-1088, 2017/11/01/ 2017.
[138] H. Fisgativa, A. Tremier, and P. Dabert, "Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion," *Waste Manag,* vol. 50, pp. 264-74, Apr 2016.
[139] M. Zheng, M. Zheng, Y. Wu, H. Ma, and K. Wang, "Effect of pH on types of acidogenic fermentation of fruit and vegetable wastes," *Biotechnology and Bioprocess Engineering,* vol. 20, no. 2, pp. 298-303, 2015/04/01 2015.
[140] D.-H. Kim, S.-H. Kim, K.-W. Jung, M.-S. Kim, and H.-S. Shin, "Effect of initial pH independent of operational pH on hydrogen fermentation of food waste," *Bioresource Technology,* vol. 102, no. 18, pp. 8646-8652, 2011/09/01/ 2011.
[141] A. P. Desbois and V. J. Smith, "Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential," *Appl Microbiol Biotechnol,* vol. 85, no. 6, pp. 1629-42, Feb 2010.
[142] L. R. Jarboe, L. A. Royce, and P. Liu, "Understanding biocatalyst inhibition by carboxylic acids," *Front Microbiol,* vol. 4, p. 272, Sep 3 2013.

[143] L. A. Royce, P. Liu, M. J. Stebbins, B. C. Hanson, and L. R. Jarboe, "The damaging effects of short chain fatty acids on Escherichia coli membranes," *Appl Microbiol Biotechnol,* vol. 97, no. 18, pp. 8317-27, Sep 2013.
[144] F. Raposo, M. A. De la Rubia, V. Fernández-Cegrí, and R. Borja, "Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures," *Renewable and Sustainable Energy Reviews,* vol. 16, no. 1, pp. 861-877, 2012.
[145] T. Amani, M. Nosrati, and T. R. Sreekrishnan, "Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects," *Environmental Reviews,* Review vol. 18, no. NA, pp. 255-278, 2010.
[146] I. Valdez-Vazquez and H. M. Poggi-Varaldo, "Alkalinity and high total solids affecting H2 production from organic solid waste by anaerobic consortia," *International Journal of Hydrogen Energy,* vol. 34, no. 9, pp. 3639-3646, 2009.
[147] T. Gameiro, M. Lopes, R. Marinho, P. Vergine, H. Nadais, and I. Capela, "Hydrolytic-Acidogenic Fermentation of Organic Solid Waste for Volatile Fatty Acids Production at Different Solids Concentrations and Alkalinity Addition," *Water, Air, & Soil Pollution,* vol. 227, no. 10, 2016.
[148] P. Vergine *et al.*, "Synthetic soft drink wastewater suitability for the production of volatile fatty acids," *Process Biochemistry,* vol. 50, no. 8, pp. 1308-1312, 2015.
[149] J. Jiang, Y. Zhang, K. Li, Q. Wang, C. Gong, and M. Li, "Volatile fatty acids production from food waste: effects of pH, temperature, and organic loading rate," *Bioresour Technol,* vol. 143, pp. 525-30, Sep 2013.
[150] H. Yuan and N. Zhu, "Progress in inhibition mechanisms and process control of intermediates and by-products in sewage sludge anaerobic digestion," *Renewable and Sustainable Energy Reviews,* vol. 58, pp. 429-438, 2016.
[151] J. B. Holm-Nielsen and P. Oleskowicz-Popiel, "Process control in biogas plants," in *Biogas Handbook: Science Production and Application*, vol. 52, A. Wellinger, J. D. Murphy, D. Baxter, and R. Braun, Eds. (Woodhead Publishing Series in Energy Ser., Amsterdam, The Netherlands: Elsevier Science & Technology, 2013, pp. 228-247.
[152] R. Moletta, Y. Escoffier, F. Ehlinger, J. Coudert, and J. Leyris, "On-line atuomatic control system for monitoring an anaerobic fluidized-bed reactor: response to organic overload," *Water Sci Technol,* vol. 30, no. 12, pp. 11-20, 1995.
[153] G. Liu, R. Zhang, H. M. El-Mashad, and R. Dong, "Effect of feed to inoculum ratios on biogas yields of food and green wastes," *Bioresour Technol,* vol. 100, no. 21, pp. 5103-8, Nov 2009.
[154] J. M. B. Domingos *et al.*, "Cheese whey integrated valorisation: Production, concentration and exploitation of carboxylic acids for the production of polyhydroxyalkanoates by a fed-batch culture," *Chemical Engineering Journal,* vol. 336, pp. 47-53, 2018.
[155] B. Xiong, T. L. Richard, and M. Kumar, "Integrated acidogenic digestion and carboxylic acid separation by nanofiltration membranes for the lignocellulosic carboxylate platform," *Journal of Membrane Science,* vol. 489, pp. 275-283, 2015.
[156] K. J. Steinbusch, E. Arvaniti, H. V. Hamelers, and C. J. Buisman, "Selective inhibition of methanogenesis to enhance ethanol and n-butyrate production through acetate reduction in mixed culture fermentation," *Bioresour Technol,* vol. 100, no. 13, pp. 3261-7, Jul 2009.
[157] D. Arslan, K. J. Steinbusch, L. Diels, H. De Wever, H. V. Hamelers, and C. J. Buisman, "Selective carboxylate production by controlling hydrogen, carbon dioxide and substrate concentrations in mixed culture fermentation," *Bioresour Technol,* vol. 136, pp. 452-60, May 2013.
[158] N.-Q. Ren *et al.*, "Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production," *international j ournal of hydrogen energy,* vol. 33, pp. 4318–4324, 2008.
[159] B. Pendyala, S. R. Chaganti, J. A. Lalman, S. R. Shanmugam, D. D. Heath, and P. C. K. Lau, "Pretreating mixed anaerobic communities from different sources:

Correlating the hydrogen yield with hydrogenase activity and microbial diversity," *internationa l jo urnal of hydrogen energy* vol. 37, pp. 12175-12186, 2012.
[160] M. Roghair *et al.*, "Effect of n-Caproate Concentration on Chain Elongation and Competing Processes," *ACS Sustain Chem Eng,* vol. 6, no. 6, pp. 7499-7506, Jun 4 2018.
[161] K. W. Golub, A. D. Smith, E. B. Hollister, T. J. Gentry, and M. T. Holtzapple, "Investigation of intermittent air exposure on four-stage and one-stage anaerobic semi-continuous mixed-acid fermentations," *Bioresour Technol,* vol. 102, no. 8, pp. 5066-75, Apr 2011.
[162] G. Giovannini, A. Donoso-Bravo, D. Jeison, R. Chamy, G. Ruız-Filippi, and A. V. Wouwer, "A review of the role of hydrogen in past and current modelling approaches to anaerobic digestion processes," *international journal of hydrogen energy 41* vol. 41, pp. 17713-17722, 2016.
[163] S. R. Harper and F. G. Pohland, "Recent developments in hydrogen management during anaerobic biological wastewater treatment," *Biotechnology and Bioengineering,* vol. 28, pp. 585-602, 1986.
[164] S. de Kok, J. Meijer, M. C. van Loosdrecht, and R. Kleerebezem, "Impact of dissolved hydrogen partial pressure on mixed culture fermentations," *Appl Microbiol Biotechnol,* vol. 97, no. 6, pp. 2617-25, Mar 2013.
[165] K. J. Steinbusch, H. V. Hamelers, and C. J. Buisman, "Alcohol production through volatile fatty acids reduction with hydrogen as electron donor by mixed cultures," *Water Res,* vol. 42, no. 15, pp. 4059-66, Sep 2008.
[166] F. Zhang *et al.*, "Fatty acids production from hydrogen and carbon dioxide by mixed culture in the membrane biofilm reactor," *Water Res,* vol. 47, no. 16, pp. 6122-9, Oct 15 2013.
[167] S. Venkata Mohan, "Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: Process evaluation towards optimization☆," *International Journal of Hydrogen Energy,* vol. 34, no. 17, pp. 7460-7474, 2009.
[168] A. Pauss, G. Andre, M. Perrier, and S. R. Guiot, "Liquid-to-Gas Mass Transfer in Anaerobic Processes: Inevitable Transfer Limitations of Methane and Hydrogen in the Biomethanation Process," *Applied and Environmental Microbiology,* vol. 56, no. 6, pp. 1636-1644, 1990.
[169] H. Yesil, A. E. Tugtas, A. Bayrakdar, and B. Calli, "Anaerobic fermentation of organic solid wastes: volatile fatty acid production and separation," *Water Sci Technol,* vol. 69, no. 10, pp. 2132-8, 2014.
[170] M. Roghair, D. P. B. T. B. Strik, K. J. J. Steinbusch, R. A. Weusthuis, M. E. Bruins, and C. J. N. Buisman, "Granular sludge formation and characterization in a chain elongation process," *Process Biochemistry,* vol. 51, no. 10, pp. 1594-1598, 2016.
[171] J. Xu, J. J. Guzman, S. J. Andersen, K. Rabaey, and L. T. Angenent, "In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis," *Chem Commun (Camb),* vol. 51, no. 31, pp. 6847-50, Apr 21 2015.
[172] O. X. Cordero and M. S. Datta, "Microbial interactions and community assembly at microscales," *Curr Opin Microbiol,* vol. 31, pp. 227-234, Jun 2016.
[173] K. Choi, B. S. Jeon, B. C. Kim, M. K. Oh, Y. Um, and B. I. Sang, "In situ biphasic extractive fermentation for hexanoic acid production from sucrose by Megasphaera elsdenii NCIMB 702410," *Appl Biochem Biotechnol,* vol. 171, no. 5, pp. 1094-107, Nov 2013.
[174] B. S. Jeon, C. Moon, B.-C. Kim, H. Kim, Y. Um, and B.-I. Sang, "In situ extractive fermentation for the production of hexanoic acid from galactitol by Clostridium sp. BS-1," *Enzyme and Microbial Technology,* vol. 53, no. 3, pp. 143-151, 2013/08/15/ 2013.
[175] F. A. Roddick and M. L. Britz, "Production of hexanoic acid by free and immobilised cells of Megasphaera elsdenii: Influence of in-situ product removal using ion exchange resin," *Journal of Chemical Technology and Biotechnology,* vol. 69, no. 3, pp. 383-391, 1997.

[176] S. Taco Vasquez, J. Dunkleman, S. K. Chaudhuri, A. Bond, and M. T. Holtzapple, "Biomass conversion to hydrocarbon fuels using the MixAlco™ process at a pilot-plant scale," *Biomass and Bioenergy,* vol. 62, pp. 138-148, 2014.
[177] Chaincraft. (30/07/2020). *ChainCraft: What if waste can become a new product?* Available: https://www.chaincraft.nl/
[178] E. de Jong and G. Jungmeier, "Biorefinery Concepts in Comparison to Petrochemical Refineries," in *Industrial Biorefineries & White Biotechnology*: Elsevier, 2015, pp. 3-33.
[179] P. Cheali, J. A. Posada, K. V. Gernaey, and G. Sin, "Economic risk analysis and critical comparison of optimal biorefinery concepts," *Biofuels, Bioproducts and Biorefining,* vol. 10, no. 4, pp. 435-445, 2016.
[180] G. W. Park *et al.*, "Volatile fatty acids derived from waste organics provide an economical carbon source for microbial lipids/biodiesel production," *Biotechnol J,* vol. 9, no. 12, pp. 1536-46, Dec 2014.
[181] K. Amulya, S. Jukuri, and S. Venkata Mohan, "Sustainable multistage process for enhanced productivity of bioplastics from waste remediation through aerobic dynamic feeding strategy: Process integration for up-scaling," *Bioresour Technol,* vol. 188, pp. 231-9, 2015.
[182] D. Pant *et al.*, "Integrated conversion of food waste diluted with sewage into volatile fatty acids through fermentation and electricity through a fuel cell," *Environ Technol,* vol. 34, no. 13-16, pp. 1935-45, Jul-Aug 2013.
[183] X.-X. Lu, G.-H. Li, S. Tao, T. Bosma, and J. Gerritse, "Volatile Fatty Acids as Electron Donors for the Reductive Dechlorination of Chloroethenes," *Journal of Environmental Science and Health, Part A,* vol. 37, no. 4, pp. 439-449, 2002.

2.2. Supplementary information on published literature review

Where literature values for concentration or rate are expressed in terms of mass, molarity, or carbon molarity, there were converted to COD (gCOD L^{-1} or gCOD L^{-1} d^{-1}) according to the conversion constants given in Table S2-1. Where synthetic or supplemented feedstocks were employed, but overall feedstock COD was omitted, a value was estimated by summing COD concentrations of the individual compounds in the substrate. When OLR data were unreported, these were calculated by dividing feedstock COD concentration by the retention time, or by multiplying by feed flow rates, depending which values were available. Similarly, MCCA production rates are calculated via the MCCA concentration in the reactor outlet. Where retention times were un-reported, these were estimated from OLR and feedstock concentrations. Yields were calculated as the ratio of the product MCCA concentration, expressed as COD, to the COD concentration of the reactor inlet.

Table S2-1 *Conversion constants used to convert reported values to corresponding COD content.*

Compound	Acronym	Formula	MW (g mol^{-1})	Ox (-)	C (mol C)
Gas					
Methane	CH_4	CH_4	16	2.0	1
Carbon dioxide	CO_2	CO_2	44	0.0	1
Hydrogen	H_2	H_2	2	0.5	0
Carboxylic acids					
Formic acid	C1	CH_2O_2	46	0.5	1
Acetic acid	C2	$C_2H_4O_2$	60	2.0	2
Propionic acid	C3	$C_3H_6O_2$	74	3.5	3
n-Butyric acid	C4	$C_4H_8O_2$	88	5.0	4
n-Valeric acid	C5	$C_5H_{10}O_2$	102	6.5	5
n-Caproic acid	C6	$C_6H_{12}O_2$	116	8.0	6
n-Heptanoic acid	C7	$C_7H_{14}O_2$	130	9.5	7
n-Caprylic acid	C8	$C_8H_{16}O_2$	144	11.0	8
n-Nonanoic acid	C9	$C_9H_{18}O_2$	158	12.5	9
Substrates					
Lactic acid	Lact	$C_3H_6O_3$	90	3.0	3
Ethanol	EtOH	C_2H_6O	46	3.0	2
Glucose	Gluc	$C_6H_{12}O_6$	180	6.0	6
Glycerol	Glyc	$C_3H_8O_3$	92	3.5	3
Sucrose	Suc	$C_{12}H_{22}O_{11}$	342	12.0	12

MW: molecular weight; Ox: oxidation reaction stoichiometry; C: carbon atoms

2.3. Literature update

Since publication of the review paper and before submission of this thesis, some noteworthy advances in the field were made and summarized in the following section.

New opportunities have emerged in terms of substrate and product spectrum and selection of inoculum for chain elongation. Additional substrates include carbon monoxide from syngas [1], glycerol from biodiesel production [2], xylose [3], leachate from municipal solid waste [4] or acidified sewage sludge [5, 6]. In addition, studies on the effects of storage and pretreatment of the feedstock on chain elongation have emerged [7, 8]. The potential product spectrum has expanded following reported selective production of isomerised or branched carboxylic acids, e.g., iso-butyrate and iso-caproate [9-11]. A direct comparison between effluent from anaerobic digestion, a caproic acid-producing reactor and animal faeces showed that all these environments house relatives to the renowned chain elongator *C. kluyveri*, yet their suitability as inoculum depends on the operational pH [12].

The research community reached a further understanding on competitive interactions to chain elongation in MMC. For instance, further reports have confirmed that bacteria performing lactate reduction to propionate outcompete lactate-based chain-elongating *Caproiciproducens* spp. at pH 6.0 and above [13]. Also butyrate-producing bacteria have been reported to take over in a long-term fermentation experiment fed with xylan and lactic acid resulting in a crash of caproic acid production [14]. Understanding these interactions between different trophic groups and metabolic processes is crucial to find the operational strategy needed to steer the community towards a selective and stable functionality.

The initial literature review listed certain concepts that required further study and provided some prospects. These have since then seen further advances. For instance, the beneficial influence of H_2 in the headspace on chain elongation has been studied further and showed to enhance electron efficiency and electron transfer capacity [15]. Two research groups have demonstrated the improved chain elongation potential of using granular fermentation, each for different complex feedstock, mainly solid-free [16, 17]. Others have further looked at alleviating product toxicity by applying biochar or anion-exchange resins [18, 19]. In the literature review, we suggested a potential pH-stabilising effect of lactic acid mediated-chain elongation. Contreras-Dávila et al. has since successfully demonstrated this concept in sequential batch fermentation of food waste where the initial build-up of lactic acid was followed by chain elongation, and thereby combated acidification by reducing available protons [20].

More studies are including omics approaches, mostly 16s rRNA gene sequencing analysis, to elucidate the MMC composition. Certain studies have included complementary types of community analysis providing a more robust discussion. For instance, flow cytometric fingerprinting statistically confirmed two distinct communities when operating at pH 5.5 or pH 7, while 16S rRNA gene-based fingerprinting did not allow to make this distinction due to statistical limitations of the data [12]. Flow cytometric analysis also enabled analysis at faster and shorter intervals of a lactate-based chain elongation community, thereby revealing a highly dynamic community, in contrast to the 16s rRNA sequencing analysis that found an unchanged core community [21]. By including fluorescence quantitative PCR the growth of *C. kluyveri* could be specifically followed in an ethanol-supplemented mixed culture fermentation of fruit and vegetable waste, showing how chain elongation took place predominantly during the middle and late stages of growth [22]. Other research labs have provided the field with the full genome sequences of newly isolated chain elongating species [23]. In addition, new metabolic models have been developed that can be used as tools to improve understanding of the various microbial roles in chain elongating MMC [24].

Advances have also been made on integrating chain elongation as a technology within a larger economic and bio-refinery context. Chwialkowska et al. included a brief techno-economic model to evaluate the economic viability of their work on caproic acid production from supplemented acid whey fermentation with consecutive liquid-liquid extraction [25]. Hu et al. build a supply chain framework for recovery of biogas, n-caproic and n-caprylic acid from organic waste and found that the combined product generation, i.e., a hybrid recovery system, allowed optimal environmental and economic benefits [26]. Carvajal-Arroyo et al. have scaled up the production of MCCA from thin stillage and their separation through a 2-compartment membrane electrolysis cells into oil at a kilogram scale [27].

References of literature update

[1] C. Liu *et al.*, "CO as electron donor for efficient medium chain carboxylate production by chain elongation: Microbial and thermodynamic insights," *Chemical Engineering Journal,* vol. 390, 2020.

[2] S. T. S. Veras *et al.*, "Anaerobic production of valeric acid from crude glycerol via chain elongation," *International Journal of Environmental Science and Technology,* vol. 17, no. 3, pp. 1847-1858, 2019.

[3] D. K. Qian *et al.*, "Caproate production from xylose by mesophilic mixed culture fermentation," *Bioresour Technol,* vol. 308, p. 123318, Jul 2020.

[4] L. Saadoun *et al.*, "Potential of medium chain fatty acids production from municipal solid waste leachate: Effect of age and external electron donors," *Waste Manag,* Oct 28 2020.

[5] Q. Wu, X. Feng, Y. Chen, M. Liu, and X. Bao, "Continuous medium chain carboxylic acids production from excess sludge by granular chain-elongation process," *J Hazard Mater,* vol. 402, p. 123471, Jul 15 2020.

[6] Q. Wang *et al.*, "Chain elongation performances with anaerobic fermentation liquid from sewage sludge with high total solid as electron acceptor," *Bioresour Technol,* vol. 306, p. 123188, Mar 14 2020.
[7] A. Duber, R. Zagrodnik, J. Chwialkowska, W. Juzwa, and P. Oleskowicz-Popiel, "Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation," *Sci Total Environ,* vol. 728, p. 138814, Aug 1 2020.
[8] H. Ma *et al.*, "Effect of ultrasonic pretreatment on chain elongation of saccharified residue from food waste by anaerobic fermentation," *Environmental Pollution,* 2020.
[9] K. D. de Leeuw, C. J. N. Buisman, and D. Strik, "Branched Medium Chain Fatty Acids: Iso-Caproate Formation from Iso-Butyrate Broadens the Product Spectrum for Microbial Chain Elongation," *Environ Sci Technol,* vol. 53, no. 13, pp. 7704-7713, Jul 2 2019.
[10] W. S. Chen, S. Huang, C. M. Plugge, C. J. N. Buisman, and D. Strik, "Concurrent use of methanol and ethanol for chain-elongating short chain fatty acids into caproate and isobutyrate," *J Environ Manage,* vol. 258, p. 110008, Mar 15 2020.
[11] S. M. de Smit, K. D. de Leeuw, C. J. N. Buisman, and D. Strik, "Continuous n-valerate formation from propionate and methanol in an anaerobic chain elongation open-culture bioreactor," *Biotechnol Biofuels,* vol. 12, p. 132, 2019.
[12] P. Candry, S. Huang, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Enrichment and characterisation of ethanol chain elongating communities from natural and engineered environments," *Sci Rep,* vol. 10, no. 1, p. 3682, Feb 28 2020.
[13] P. Candry, L. Radic, J. Favere, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Mildly acidic pH selects for chain elongation to caproic acid over alternative pathways during lactic acid fermentation," *Water Res,* vol. 186, p. 116396, Sep 7 2020.
[14] B. Liu, S. Kleinsteuber, F. Centler, H. Harms, and H. Strauber, "Competition Between Butyrate Fermenters and Chain-Elongating Bacteria Limits the Efficiency of Medium-Chain Carboxylate Production," *Front Microbiol,* vol. 11, p. 336, 2020.
[15] Q. Wu *et al.*, "Concentrating lactate-carbon flow on medium chain carboxylic acids production by hydrogen supply," *Bioresour Technol,* vol. 291, p. 121573, Nov 2019.
[16] J. M. Carvajal-Arroyo *et al.*, "Granular fermentation enables high rate caproic acid production from solid-free thin stillage," *Green Chemistry,* vol. 21, no. 6, pp. 1330-1339, 2019.
[17] Q. Wu, X. Feng, W. Guo, X. Bao, and N. Ren, "Long-term medium chain carboxylic acids production from liquor-making wastewater: Parameters optimization and toxicity mitigation," *Chemical Engineering Journal,* vol. 388, 2020.
[18] Y. Liu, P. He, W. Han, L. Shao, and F. Lü, "Outstanding reinforcement on chain elongation through five-micrometer-sized biochar," *Renewable Energy,* vol. 161, pp. 230-239, 2020.
[19] J. Yu *et al.*, "Enhanced Anaerobic Mixed Culture Fermentation with Anion-Exchange Resin for Caproate Production," *Processes,* vol. 7, no. 7, 2019.
[20] C. A. Contreras-Davila, V. J. Carrion, V. R. Vonk, C. N. J. Buisman, and D. Strik, "Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation," *Water Res,* vol. 169, p. 115215, Feb 1 2020.
[21] J. Lambrecht *et al.*, "Key sub-community dynamics of medium-chain carboxylate production," *Microb Cell Fact,* vol. 18, no. 1, p. 92, May 28 2019.
[22] J. Yu *et al.*, "Performance and microbial characterization of two-stage caproate fermentation from fruit and vegetable waste via anaerobic microbial consortia," *Bioresour Technol,* vol. 284, pp. 398-405, Jul 2019.
[23] B. Liu, D. Popp, H. Strauber, H. Harms, and S. Kleinsteuber, "Draft Genome Sequences of Three Clostridia Isolates Involved in Lactate-Based Chain Elongation," *Microbiol Resour Announc,* vol. 9, no. 32, Aug 6 2020.

[24] M. J. Scarborough, J. J. Hamilton, E. A. Erb, T. J. Donohue, and D. R. Noguera, "Diagnosing and Predicting Mixed-Culture Fermentations with Unicellular and Guild-Based Metabolic Models," *mSystems,* vol. 5, no. 5, Sep 29 2020.
[25] J. Chwialkowska, A. Duber, R. Zagrodnik, F. Walkiewicz, M. Lezyk, and P. Oleskowicz-Popiel, "Caproic acid production from acid whey via open culture fermentation - Evaluation of the role of electron donors and downstream processing," *Bioresour Technol,* vol. 279, pp. 74-83, May 2019.
[26] Y. Hu, M. Scarborough, H. Aguirre-Villegas, R. A. Larson, D. R. Noguera, and V. M. Zavala, "A Supply Chain Framework for the Analysis of the Recovery of Biogas and Fatty Acids from Organic Waste," *ACS Sustainable Chemistry & Engineering,* vol. 6, no. 5, pp. 6211-6222, 2018.
[27] J. M. Carvajal-Arroyo, S. J. Andersen, R. Ganigué, R. A. Rozendal, L. T. Angenent, and K. Rabaey, "Production and extraction of medium chain carboxylic acids at a semi-pilot scale," *Chemical Engineering Journal,* 2020.

2.4. Concluding remarks

To address the first thesis goal, the literature review provided an overview on the knowledge available regarding chain elongation in MMC fermentation of complex organic feedstock. This naturally led to addressing the second goal, identifying operational strategies to stimulate chain elongation in the fermentation of a complex feedstock. For instance, it can be concluded from the literature study that operating at slightly acidic pH (between 5.0-6.0) and starting operation at an organic overload, a F/M of 5 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$ or more, are proven strategies to steer an AD microbiome towards accumulation of VFA, and potentially MCCA. However, it remained unclear what to choose for certain key operational parameters, such as how to provide a stimulus of an organic overload over long-term operation or which HRT or OLR would be sufficient to ensure hydrolysis of the complex food waste and accumulate chain elongation precursors.

It was suggested in the literature review that a single-stage reactor would have to be operated in a way that stimulates the *in situ* production of VFA and electron donors such as ethanol, lactic acid and H_2. We hypothesised operating semi-continuously, thus longer fermentation cycles, could stimulate the accumulation of chain elongation precursors more than continuous feeding.

Lastly, the literature review stated that food waste is a promising feedstock for MCCA. However, limited work has been done on chain elongation in mixed food waste without supplementation of electron donors. Furthermore, for the application of resource recovery in recycling facilities, it is necessary to know how variability of the feedstock affects product outcome.

2.5. Research objectives

The first thesis goal, which is acquiring knowledge regarding chain elongation in mixed culture fermentation of complex organic feedstock, was mainly addressed within the literature review (Chapter 2).

In order to address the second goal, i.e., identifying operational strategies that enable chain elongation in food waste fermentation with minimal chemical addition in a simple STR, a clear set of research objectives was defined following the knowledge acquired in the literature study:

1. Explore overloading a single-stage reactor with food waste as operational strategy to steer a MMC performing AD towards chain elongation in long-term operation (Chapter 3)
2. Evaluate how OLR and HRT influence food waste fermentation in the context of MCCA production (Chapter 4)
3. Assess the effect of a semi-continuous feeding pattern on the production of MCCA (Chapter 5)
4. Monitor feedstock composition and its impact on the fermentation outcome (Chapter 6)

The third thesis goal is investigating the underlying mechanisms that steer MMC fermentation of food waste to MCCA production. To address it, two additional horizontal objectives were defined:

5. Study the composition of the microbial communities that are formed in bioreactors under different operating conditions to relate the different microbial groups to a type of fermentation (addressed in Chapters 3 to 5)
6. Characterise the fermentation pathways in the production of MCCA and other AF products through cycle studies in the different MMC systems (addressed in Chapters 3 to 6).

Chapter 3. Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation

It is valuable to demonstrate MCCA production in a simple setup similar to AD in order to ease integration within existing waste treatment facilities. Thus, in line with the scope of the thesis, this first research chapter targeted chain elongation in food waste fermentation using a single-phase stirred tank reactor with minimal chemical supplementation. Only sodium hydroxide was dosed to prevent acid inhibition.

This chapter tackled the first research objectives concluded from the literature review. An organic overload was evaluated as operational strategy in long-term operation to shift fermentation from anaerobic digestion to chain elongation. Reactor performance was assessed in combination with the underlying fermentative pathway and microbial community structure to also address the fifth and sixth research objective.

Reactor operation was performed during the first, introductory year of the integrated PhD (one year MRes and three years PhD). The data on reactor performance was partially processed for the MRes qualification. For this thesis and further publication, it was supplemented with further data processing, microbial community analysis and a deepened discussion of the results and its impact.

This chapter is submitted in an alternative thesis format in line with Appendix 6A of the “Specifications for Higher Degree Theses and Portfolios” as required by the University of Bath. This work was published in MDPI Processes’ Special Issue "Anaerobic Digestion for Bioenergy and Biochemicals Production":

V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting Organic Load as a Strategy to Direct Single-Stage Food Waste Fermentation from Anaerobic Digestion to Chain Elongation," Processes, vol. 8, no. 11, 2020. doi:10.3390/pr8111487

The dataset was made available as:

V. De Groof, M. Coma Bech, D. J. Leak, T. Arnot, and A. Lanham, "Dataset for "Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation"," ed. Bath: University of Bath Research Data Archive, 2020. doi: 10.15125/BATH-00941.

Author Contributions: Conceptualization, M.C.; methodology, V.D.G. and M.C.; investigation, V.D.G. and M.C.; data curation, V.D.G.; writing—original draft preparation, V.D.G.; writing—review and editing, M.C., T.C.A., D.J.L., A.B.L. and V.D.G.; supervision, M.C., T.C.A., D.J.L. and A.B.L.; project administration, T.C.A. and A.B.L.; funding acquisition, T.C.A. All authors have read and agreed to the published version of the manuscript.

This declaration concerns the article entitled:			
Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation			
Publication status (tick one)			
~~**Draft manuscript**~~ ~~**Submitted**~~ ~~**In review**~~ ~~**Accepted**~~ **Published**			
Publication details (reference)	V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting Organic Load as a Strategy to Direct Single-Stage Food Waste Fermentation from Anaerobic Digestion to Chain Elongation," Processes, vol. 8, no. 11, 2020. doi:10.3390/pr8111487		
Copyright status (tick the appropriate statement)			
I hold the copyright for this material ~~Copyright is retained by the publisher, but I have been given permission to replicate the material here~~			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: The candidate contributed considerably to the formulation of ideas. Initial conceptualization of the experiment was done by Marta Coma. In discussion with all co-authors, the candidate formulated the idea further into the final published outcome – 65% Design of methodology: The candidate considerably contributed to design of methodology in collaboration with co-author Marta Coma. – 65% Experimental work: The candidate predominantly executed the experimental work and data curation. – 80% Presentation of data in journal format: The candidate wrote the original draft of the paper. Co-authors reviewed and proposed edits. – 70%		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	Vicky De Groof	**Date**	26th March 2021

3.1. Published research article: Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation

Vicky De Groof [1,4], Marta Coma [3], Tom Arnot [2,3,4], David J. Leak [3,4,5], Ana B. Lanham [2,3,4]*

[1] EPSRC Centre for Doctoral Training in Sustainable Chemical Technologies, University of Bath, Claverton Down, Bath BA2 7AY, UK; V.De.Groof@bath.ac.uk

[2] Water Innovation & Research Centre (WIRC), University of Bath, Claverton Down, Bath BA2 7AY, UK T.C.Arnot@bath.ac.uk (T.C.A); D.J.Leak@bath.ac.uk (D.J.L)

[3] Centre for Sustainable and Circular Technologies (CSCT), University of Bath, Claverton Down, Bath BA2 7AY, UK; M.Coma@bath.ac.uk

[4] Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK

[5] Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

***** Correspondence: A.Lanham@bath.ac.uk; Tel.: +441225384544

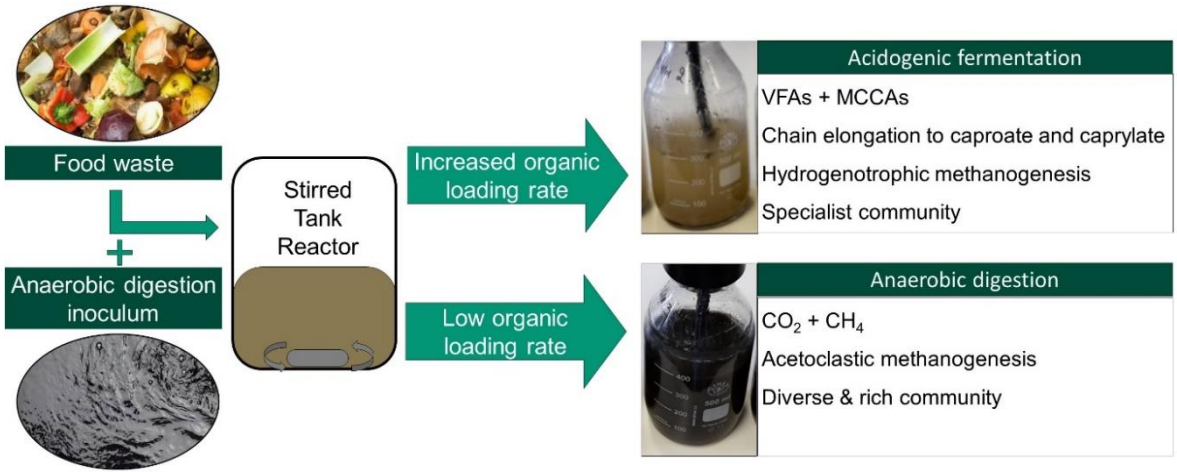
Received: 22 October 2020; Accepted: 14 November 2020; Published: 18 November 2020

Abstract

Production of medium chain carboxylic acids (MCCA) as renewable feedstock bio-chemicals, from food waste (FW), requires complicated reactor configurations and supplementation of chemicals to achieve product selectivity. This study evaluated the manipulation of organic loading rate in an un-supplemented, single-stage stirred tank reactor to steer an anaerobic digestion (AD) microbiome towards acidogenic fermentation (AF), and thence to chain elongation. Increasing substrate availability by switching to a FW feedstock with a higher COD stimulated chain elongation. The MCCA species n-caproic (10.1 ± 1.7 g L^{-1}) and n-caprylic (2.9 ± 0.8 g L^{-1}) acid were produced at concentrations comparable to more complex reactor set-ups. As a result, of the adjusted operating strategy, a more specialised microbiome developed containing several MCCA-producing bacteria, lactic acid-producing *Olsenella* spp. and hydrogenotrophic methanogens. By contrast, in an AD reactor that was operated in parallel to produce biogas, the retention times had to be

doubled when fed with the high-COD FW to maintain biogas production. The AD microbiome comprised a diverse mixture of hydrolytic and acidogenic bacteria, and acetoclastic methanogens. The results suggest that manipulation of organic loading rate and food-to-microorganism ratio may be used as an operating strategy to direct an AD microbiome towards AF, and to stimulate chain elongation in FW fermentation, using a simple, un-supplemented stirred tank set-up. This outcome provides the opportunity to repurpose existing AD assets operating on food waste for biogas production, to produce potentially higher value MCCA products, via simple manipulation of the feeding strategy.

Graphical abstract



Keywords

Acidogenic fermentation

Anaerobic digestion

Food waste

Medium chain carboxylic acid

Microbial chain elongation

Mixed culture

Organic loading rate

3.1.1. Introduction

To achieve a sustainable and circular bio-economy, it is crucial to minimise food waste (FW, 88M tonnes in EU annually) and use the unavoidable, inedible fraction as feedstock for the production of bio-chemicals [1, 2]. FW is rich in carbon, nutrients and moisture, making it a favourable substrate for mixed microbial culture fermentation, such as anaerobic digestion (AD) [3]. Recently, research has focused on the carboxylate platform where the liquid intermediates formed during the primary acidogenic fermentation (AF) steps of AD are targeted to generate products with a higher value than biogas [4]. Amongst the different compounds that can be obtained, medium-chain carboxylic acids (MCCA) are of particular interest due to their lower water solubility, which facilitates their recovery, their antimicrobial properties, and their potential application as platform chemical or liquid drop-in biofuels [5-7].

Selective operational conditions in AF allow to direct the product outcome of FW towards, e.g., volatile fatty acids (VFA) [8, 9], lactic acid [10, 11] or hydrogen [12, 13]. Some bacteria in AF can elongate short VFA into MCCA with 6 (n-caproic acid) to 8 (n-caprylic acid) carbon atoms via the reversed β-oxidation pathway [14]. Selectivity towards chain elongation is subject to the absence of competitive pathways and the availability of electron donors such as hydrogen, lactic acid or ethanol [15]. External addition of electron donors is generally not desirable as it has associated costs and a negative impact on the environmental life cycle assessment of waste fermentation for MCCA production [16]. Finding the most suitable operational parameters to direct the complex network of biochemical reactions in mixed culture fermentation towards chain elongation is still a topic of research.

One of the factors currently limiting MCCA yields is competition with methane generation, i.e., transformation of soluble organics into gaseous products. Strategies proposed to block methanogenesis, without the addition of chemical inhibitors, include inoculum pre-treatment by heat shock to select for spore-forming bacteria [17, 18] which are mainly fermentative, or lowering the sludge retention time (SRT) to wash out methanogens [8]. Additionally, methanogens are generally more sensitive to a lower pH (< 6.0) and the accumulation of VFA that follows from an organically overloaded bioreactor [19]. Organic overload can be obtained in batch operation by increasing the loading rate or food-to-microorganism ratio (F/M > 1 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$)[20].

At low pH and high product concentrations, the carboxylate product species shift towards their acidic, undissociated forms. These acidic compounds have antimicrobial properties and can slow down or inhibit metabolism [21]; hence, chain elongation has been improved by alleviating product toxicity via *in situ* extraction of MCCA [22-24]. Elongation is also

improved by operating at low hydraulic retention time (HRT) and thus low product concentrations, whilst maintaining production rates, in systems with biomass retention, such as in an up-flow anaerobic sludge blanket reactor [25], or granular sludge reactors [26, 27]. Some of these solutions require high recirculation flow rates, which can prove challenging for substrates with high solid concentrations (e.g., >6% *w/w* total solids), such as FW. Alternatively, MCCA production from these types of feedstock has been improved by using two-stage systems. Hydrolysis and acidogenesis can be optimised in a separate bioreactor to chain elongation [28-30], or by using leach-bed reactors where soluble, inhibitory monomers are removed from the solid substrate [31]. Recent work has found hydrothermal and ultrasonic pre-treatment of FW can enhance MCCA production in fermentation [32]. Such adaptions are a trade-off between achieving higher yields and the costs related to more complicated operation and reactor design. For these reasons, AD reactor configurations at a commercial scale in waste valorisation facilities are typically single-step systems, such as single-stage stirred-tank reactors (STR), as they allow simpler processing and lower investment costs [33, 34]. It is, therefore, valuable to explore the potential of such simpler reactor setups for MCCA production from FW to facilitate commercial implementation.

Lab-scale trials have demonstrated that, for maize and switch grass stillage as feedstock, long-term MCCA formation can be achieved in STRs, without the need for addition of electron donors [35, 36]. This was due to the *in-situ* production of lactic acid as electron donor during the fermentation process. A similar mechanism was found for short-term sequential batch fermentation of FW [37]. However, the long-term conversion of FW to MCCA in a STR setup without electron donor supplementation has yet to be demonstrated. In addition, these studies inoculated their reactors with enriched microbiomes and hence the strategy required to operationally transform functionality from a microbiome performing AD to one performing chain elongation remains largely unknown. Therefore, the aim was to demonstrate how an AD microbiome fermenting FW in a simple STR can be redirected, during long-term operation towards either biogas or MCCA production, by solely changing operating conditions, predominantly organic overload, i.e., without addition of methanogenic inhibitors or electron donors. This simple approach allows repurposing of existing food waste AD assets to produce higher value products (MCCA), which is an attractive means for accelerating the deployment of circular economy practices. To allow direct comparison of functionality and microbial community development, two STRs, one for AD and the other for AF, were operated in parallel with the same inoculum and fed with the same FW substrate.

3.1.2. Materials and methods

Food waste (FW) and inoculum were sourced from a full-scale industrial AD plant (GENeco, Bristol, UK). The FW in the AD plant comprises packaged and unpackaged Category 3 FW collected from households, supermarkets, restaurants and other catering services, and is ground and mixed with a variety of liquid streams from the food-processing industry and/or the liquid fraction of anaerobic digester effluent, to form a slurry-like mixture. Two batches of FW slurry were collected one month apart (FW 1 and FW 2). Upon collection each batch of FW was characterised (Table 3-1) and frozen in aliquots (-18 °C) for reactor feeding. The inoculum was collected from the effluent of a mesophilic continuous anaerobic digester (2,400 m^3, STR) processing pasteurised FW. The inoculum was diluted with tap water to reach a set VS in the reactors and acclimated overnight to operating temperature before initiating the feed.

Table 3-1 *Characteristics of the two food waste substrates (FW 1 and FW 2) used in this study. Analysis was performed in duplicate and presented with standard deviation.*

Parameter	**FW 1**	**FW 2**
pH	5.0 ± 0.1	5.4 ± 0.1
Conductivity (mS cm^{-1})	6.20	6.16
Solid Content (*% w/w*)		
Total Solids	9.94 ± 0.07	17.7 ± 1.6
Volatile Solids	8.832 ± 0.002	16.3 ± 1.1
Chemical Oxygen Demand (gCOD L^{-1})		
Total COD	150 ± 1	297 ± 9
Soluble COD	37.4 ± 1.1	38.2 ± 0.1
Soluble Compounds (g L^{-1})		
Acetic acid	1.27 ± 0.18	0.89 ± 0.16
n-Propionic acid	0.63 ± 0.02	0.63 ± 0.19
n-Butyric acid	<0.31	0.35 ± 0.24
MCCA (C5-C8)	0.00 ± 0.00	0.00 ± 0.00
Glucose	3.61 ± 0.14	5.03 ± 0.87
Sugar compounds*	0.55 ± 0.07	5.34 ± 2.13
Lactic acid	7.27 ± 0.34	2.48 ± 0.02
Ethanol	1.39 ± 0.71	0.80 ± 0.16

* fructose, overlap with sucrose and xylose; *% w/w*: mass fraction; MCCA (C5-C8): medium chain carboxylic acids (chain length of 5 to 8 carbon atoms).

Two 2 L STRs were operated semi-continuously (1 L working volume, magnetic stirrer mixing, 35 °C). Feeding events took place every 3.5 days, where a fixed volume of reactor effluent, determined by the set OLR, was manually replaced by the same amount of FW. One STR was set up for AD by starting operation with a F/M of 0.8 gCOD gVS^{-1} and 20 gVS L^{-1} of inoculum, feeding at an average OLR of 4.2 ± 0.4 gCOD $L^{-1}d^{-1}$ (2.5 ± 0.2 gVS L^{-1} d^{-1}). The second STR was set up for AF by organic overload at a F/M ratio of 8.4 gCOD gVS^{-1} and 5 gVS L^{-1} of inoculum, feeding at an average OLR of 8.5 gCOD L^{-1} d^{-1} (5.0 gVS L^{-1} d^{-1}). Following start-up, reactors were operated in two distinct phases according to the conditions

in Table 3-2. The pH was manually corrected to a minimum of 7.3 ± 0.1 for the AD reactor, or 6.0 ± 0.2 for the AF reactor with sodium hydroxide (1 or 2 M) after each substrate addition. Reactors were operated as airtight, but at intervals were briefly open to atmosphere during feeding and pH correction.

Table 3-2 *Overview of operational parameters for the AD and AF reactors.*

STR	Feedstock	Days	OLR (gCOD L^{-1} d^{-1})	HRT (d)
Phase 1 – Shift functionality with increased organic load				
AD	FW 1	0 – 14	4.2 ± 0.4	35 ± 3
	FW 2	14 – 32	8.5 ± 0.8	35 ± 3
AF	FW 1	0 – 14	8.5 ± 0.7	18 ± 2
	FW 2	14 – 32	17.1 ± 1.5	18 ± 2
Phase 2 – Establish longer term operation				
AD	FW 2	0 – 80	4.4 ± 0.5	69 ± 6
AF	FW 2	0 – 10	NA*	NA*
	FW 2	10 – 87	21.3 ± 1.6	14 ± 1

* Gradual start-up from Phase 1 AF after reactor pause where OLR was increased from 9.2 to 21.3 gCOD L^{-1} d^{-1} (HRT decrease from 32 to 14 days).

The F/M was determined at start-up and each point of feeding as the amount of total COD (tCOD) fed, over the volatile solids (VS) concentration in the reactors at that time (Equation (3-1)).

$$F/M_{(i)} = (C_{feed(i)} \times V_{feed(i)})/VS_{reactor(i)} \text{ [gCOD gVS}^{-1}\text{]}, \quad (3\text{-}1)$$

where i represents the feeding event, C_{feed} is the organic content expressed as tCOD in the feed in gCOD L^{-1} and V represents volume in L. The OLR was calculated as an average between feeding events as a proxy for continuous operation as the amount of total chemical oxygen demand (tCOD) fed, over the time in between feedings in days per reactor volume (Equation (3-2)).

$$OLR_{(i)} = (C_{feed(i)} \times V_{feed(i)})/(V_{reactor(i)} \times (t_{(i+1)} - t_{(i)})) = C_{feed(i)}/HRT_{(i)} \text{ [gCOD L}^{-1}\text{ d}^{-1}\text{]}, \quad (3\text{-}2)$$

where t represents the day of reactor operation, and $t_{(i+1)}$-$t_{(i)}$ is the time between feeding points.

AD performance was determined from the methane yield, i.e., volume of methane produced at Standard Temperature and Pressure (STP, 273.15 K and 100 kPa) in between feeding events over the amount of substrate fed, expressed in VS or tCOD. The performance of AF was assessed by: (i) the average net production rate (NP_{CA}), i.e., the increase of a given carboxylic acid (CA) in the effluent expressed as COD and corrected for feedstock content (Equation (3-3)); (ii) the average net yield (Y_{CA}), ,i.e., NP_{CA} over OLR (Equation (3-4)); and (iii) selectivity (S_{CA}) of carboxylic acid formation, i.e., the NP_{CA} of a specific single CA over the net production rate of all carboxylic acids expressed as COD (Equation (3-5)).

$$NP_{CA(i)} = (C_{CA(i),\ effluent} - C_{CA(i-1),\ feed})/HRT_{(i-1)}\ [gCOD\ L^{-1}\ d^{-1}], \quad (3\text{-}3)$$

$$Y_{CA(i)} = NP_{CA(i)} / OLR_{(i-1)}\ [\%], \quad (3\text{-}4)$$

$$S_{CA(i)} = NP_{CA(i)} / \sum NP_{CA(i)}\ [\%] \quad (3\text{-}5)$$

Waste stabilization was evaluated by the removal efficiency of VS (VS_{rem}) or tCOD ($tCOD_{rem}$) over the respective load (Equation 3-6).

$$VS_{rem} = (VS_{feed} - VS_{effluent})/ VS_{feed},\ tCOD_{rem} = (tCOD_{feed} - tCOD_{effluent})/ tCOD_{feed}\ [\%] \quad (3\text{-}6)$$

Total solids (TS) and VS were determined according to Standard Methods 2540G [38]. The COD was assessed with cuvette tests (LCK014, LCI400, Hach, Dusseldorf, Germany) before and after filtration (0.45 µm) for tCOD and soluble COD (sCOD), respectively.

Liquid samples were taken from the reactor effluent before each feeding event to follow process performance. Carboxylic acids (chain length 2 to 8) were measured by a method adapted from Manni and Caron (1995) using gas chromatography (GC, 7890B, Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-FFAP 122-3232 column (30 m × 0.25 mm × 0.25 m; Agilent Technologies) with a flame ionization detector (FID) [39]. Liquid samples were conditioned with sulphuric acid, sodium chloride and 2-methyl hexanoic acid as internal standard for quantification, before extraction with diethyl ether. Samples (1 µL) were injected at 250 °C with a split ratio of 10, 3 mL min^{-1} purge flow, and N_2 carrier gas at 2.4 mL min^{-1} flow rate. The oven temperature increased by 8 °C min^{-1} from 110 °C to 165 °C where it was kept for 2 min and the FID temperature was set at 300 °C. The FW samples were further characterised for ethanol, lactic acid, and sugars by high pressure liquid chromatography (HPLC, 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) as in Coma et al. [16] with the oven temperature adjusted to 65 °C.

Volumetric biogas production was evaluated by determining the displacement of acidified water (pH < 4.3, HCl) in calibrated glass columns connected to the reactor headspace and reported at STP. Biogas samples were collected from the glass columns just before effluent withdrawal and feed addition. CH_4 and CO_2 were measured by GC (7890A, Agilent Technologies, Santa Clara, CA, USA) with a HP-PLOT/Q column (Agilent Technologies, Santa Clara, CA, USA) whereby CH_4 was detected by FID and CO_2 was detected with a thermal conductivity detector (TCD)[34]. H_2, N_2 and O_2 percentages were determined by GC-TCD (3800GC, Varian, Agilent Technologies, Santa Clara, CA, USA) equipped with a molecular sieve column (13 × 60-80 mesh, 1.5 m × 1/8" x 2.0 mm) with a run time of 1 min. Injection, column, and TCD were set at 250, 40, and 200 °C, respectively. Argon was used as the carrier gas at total flow rate of 75 mL min^{-1}. Calibration was carried with multiple

injections of a mixture containing permanent gases at 1%. Gas composition was corrected for air intrusion assuming biogas produced comprised only CH_4, CO_2 and N_2 and normalised to 100%.

Biomass samples for community analysis were taken in duplicate on Day 77 from AD and Day 84 from AF reactor (Phase 2). Samples were stored at -18°C and processed by DNAsense (Aalborg, North Jutland, Denmark). In short, DNA was extracted using the FastDNA Spin kit for Soils (MP Biomedicals, Solon, OH, USA) [40]. The 16S rRNA gene region V4 sequencing libraries were prepared by an Illumina-based custom protocol [41]. PCR amplifications were done with 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3') as primer pair to cover both archaeal and bacterial domains [42]. The amplicons were paired-end sequenced (2x300 bp) on a MiSeq sequencer (Illumina, San Diego, CA, USA). Forward and reverse reads were prepared for use in the UPARSE workflow [43-45]. The reads were clustered into operational taxonomic units (OTUs) at 97% similarity. Taxonomy was assigned using the RDP classifier in QIIME (80% confidence cut-off) and the SILVA database (release 132) [46-48]. The results were analysed in R (v.3.5.1, https://www.r-project.org/, 2018) through the Rstudio IDE using the ampvis package (v.2.5.8) and the DNAsense app (DNAsense, Aalborg, North Jutland, Denmark) [40, 49]. Sequences have been deposited with the ENA database (accession number PRJEB39281). Rarefaction curves, relative abundances, alpha-diversity measures and taxonomic classifications for all samples are made available within a comprehensive dataset on the University of Bath Research Data Archive [50].

3.1.3. Results

3.1.3.1. Elevated organic load directed anaerobic digester sludge towards acidogenic fermentation

A start-up strategy of higher F/M and OLR, and hence indirectly lower HRT compared to traditional AD, led in the AF reactor to a net production of carboxylic acids with minimal biogas generation. After two weeks (less than one HRT), VFA (acetic (C2), n-propionic (C3) and n-butyric acid (C4)), accumulated to a total 19.0 gCOD L^{-1} and n-valeric (C5) and n-caproic (C6) acid to 6.1 gCOD L^{-1}. The biogas had an average composition of 77 ± 1 % CO_2, 23 ± 1 % H_2 and < 1 % CH_4, (Figure 3-1). In contrast, the operational strategy in the AD reactor resulted in conventional anaerobic digestion of the FW with a methane yield of 0.32 m^3 CH_4 $kgVS^{-1}$ (0.19 m^3 CH_4 $kgCOD^{-1}$) on Day 14. This falls within the range of values reviewed for the anaerobic digestion of FW [3].

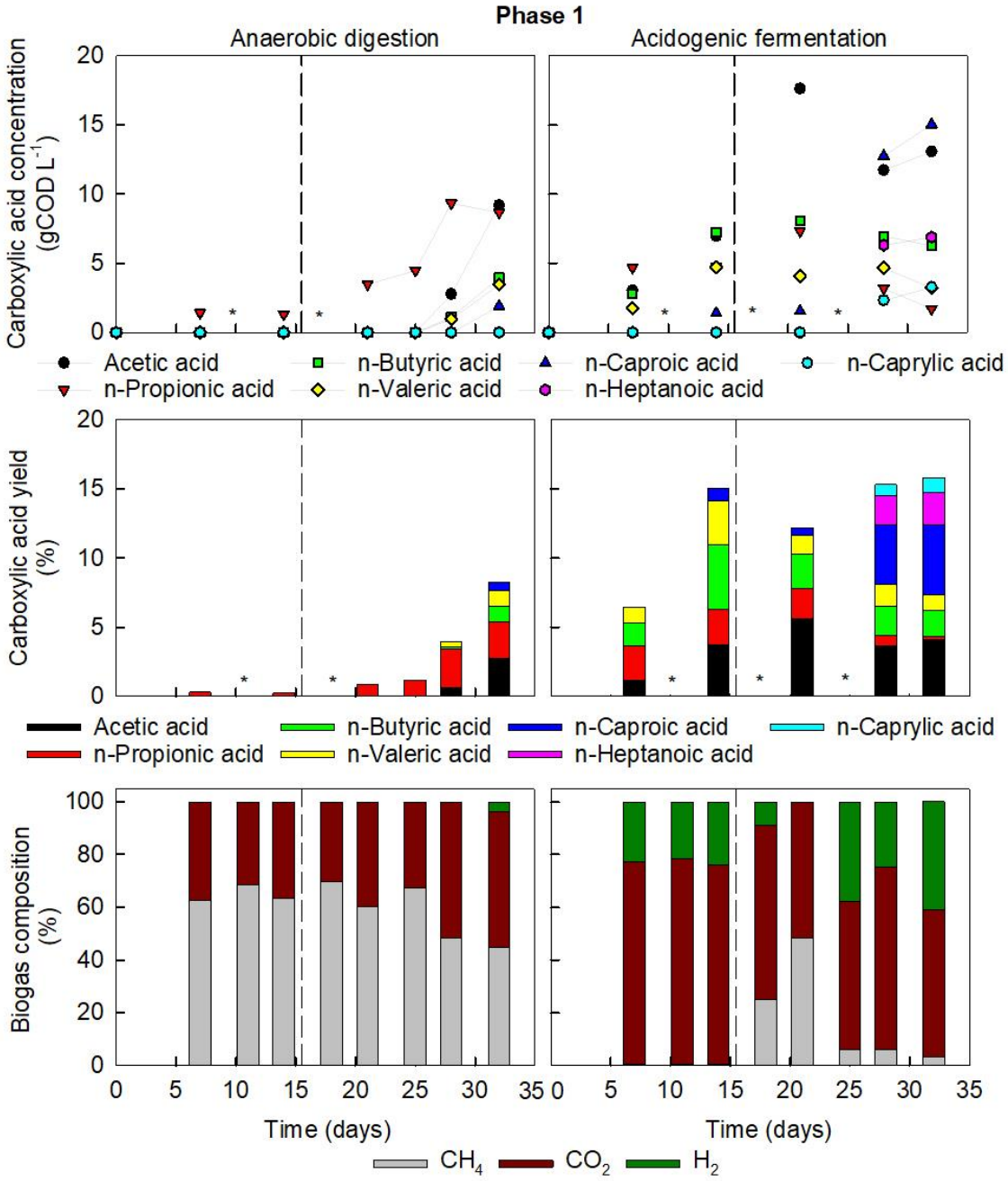


Figure 3-1 *Key chemical compounds in Phase 1 of reactor operation in AD (left) and AF (right). Concentrations (top) and yields (middle) of liquid fermentation compounds and biogas composition (bottom). Dashed lines represent change of feed from FW1 to FW2. *Not determined.*

Due to the increased COD content of FW2, the OLR doubled from Day 14 for each reactor while maintaining the HRT. The OLR increased to 17 ± 2 gCOD $L^{-1}d^{-1}$, in the AF reactor and to 8.5 ± 0.8 gCOD L^{-1} d^{-1} in the AD reactor. In the AF reactor production of carboxylic acids increased and chain elongation occurred up to caprylic acid (C8). By Day 32 MCCA (C6, heptanoic (C7) and C8) totalled 25.2 gCOD L^{-1}, i.e., 54% selectivity of all carboxylic acids (Figure 3-1). Both feedstocks, FW1 and FW2, contained electron donors for chain elongation, but with FW1 containing slightly more ethanol and nearly three times more lactic

acid than FW2. Therefore, with the shift from FW1 to FW2, the loading rate of electron donors decreased from 0.60 ± 0.09 gCOD L^{-1} d^{-1} to 0.25 ± 0.02 gCOD L^{-1} d^{-1}, nevertheless chain elongation was stimulated.

The doubling of OLR in AD increased F/M to 1.7 gCOD gVS^{-1}, i.e., higher than typical AD values (1 gCOD gVS^{-1}) [20]. After two weeks of operating at the elevated OLR, which was similar to the initial OLR of AF at start-up, the pH dropped to 6.0 in between feeding events. This caused methanogenesis to decrease, with less biogas production and a reduction of methane to 45 % of biogas composition, with a resulting yield of 0.02 m^3CH_4 $kgVS^{-1}$. Carboxylic acids accumulated simultaneously, reaching concentrations similar to those found during the start-up of AF reactor, namely 21.9 gCOD L^{-1} VFA and 5.4 gCOD L^{-1} of C5 and C6 (Figure 3-1).

Total biogas production in AF remained at least 9 times lower than the AD reactor. Despite the high organic load, some methanogenic activity did persist in AF. From Day 7 to 14, less than 1% of CH_4 was detected in the biogas, but it peaked on Day 21 to 48.4 % CH_4 (Figure 3-1). During the previous period, an increase in organic solids concentration in the reactor was observed, from 5 to 22 gVS L^{-1}, lowering F/M to 1.38 gCOD gVS^{-1} on Day 14, with a lower F/M enhancing methanogenesis. This could be due to the accumulation of substrate particles or biomass growth. By Day 32 methanogenesis subsided again to 3.1% CH_4 content in the off gas probably due to a consistent overload from FW2 with higher COD.

To verify whether methanogensis could recover, no fresh substrate was added to either reactor for 2 weeks (equivalent to 4 feeding events) and pH was corrected. The pH dropped again from 7.1 to 5.5 regardless of the absence of fresh organic material. The overall carboxylic acid content did not reduce, and methanogenic activity did not recover. The high carboxylates concentrations in AD before starvation (27.3 gCOD L^{-1}, C2-C6) continued to inhibit methanogenesis as they were far above inhibitory levels, even at neutral pH, i.e., approx. 9.5 gCOD L^{-1} [51, 52]. Similarly, in the AF reactor, methane in the biogas remained low (≈1% CH_4).

3.1.3.2. High-COD food waste required increased retention times for AD but promoted chain elongation in AF

The AD reactor was restarted similar to Phase 1 with fresh inoculum and operated in parallel as control during Phase 2 of operation. The HRT was increased to 69 ± 7 days to compensate for the increased tCOD content of the feedstock. The AD reactor was giving methane yields of 0.51 m^3 CH_4 $kgVS^{-1}$ by Day 24, which was similar to Phase 1. Thus, the AD operation could be adapted for the COD-rich substrate by moving to a longer HRT. After

56 days of operation, i.e., less than one HRT, VFA increased again peaking at 7.3 gCOD L^{-1} and methane yield reduced to 0.17 m^3 CH_4 $kgVS_{added}^{-1}$ (Figure 3-2). The increase in HRT could therefore only temporarily restore the AD functionality when applying the high COD-FW. Microbial community analysis revealed that methanogens were still present and thus, were likely inhibited by the VFA. The OLR of 4.4 ± 0.5 gCOD L^{-1} d^{-1} (2.4 ± 0.3 gVS L^{-1} d^{-1}) is near the upper limit for stable mono-digestion of FW (2.5 gVS L^{-1} d^{-1}) [3].

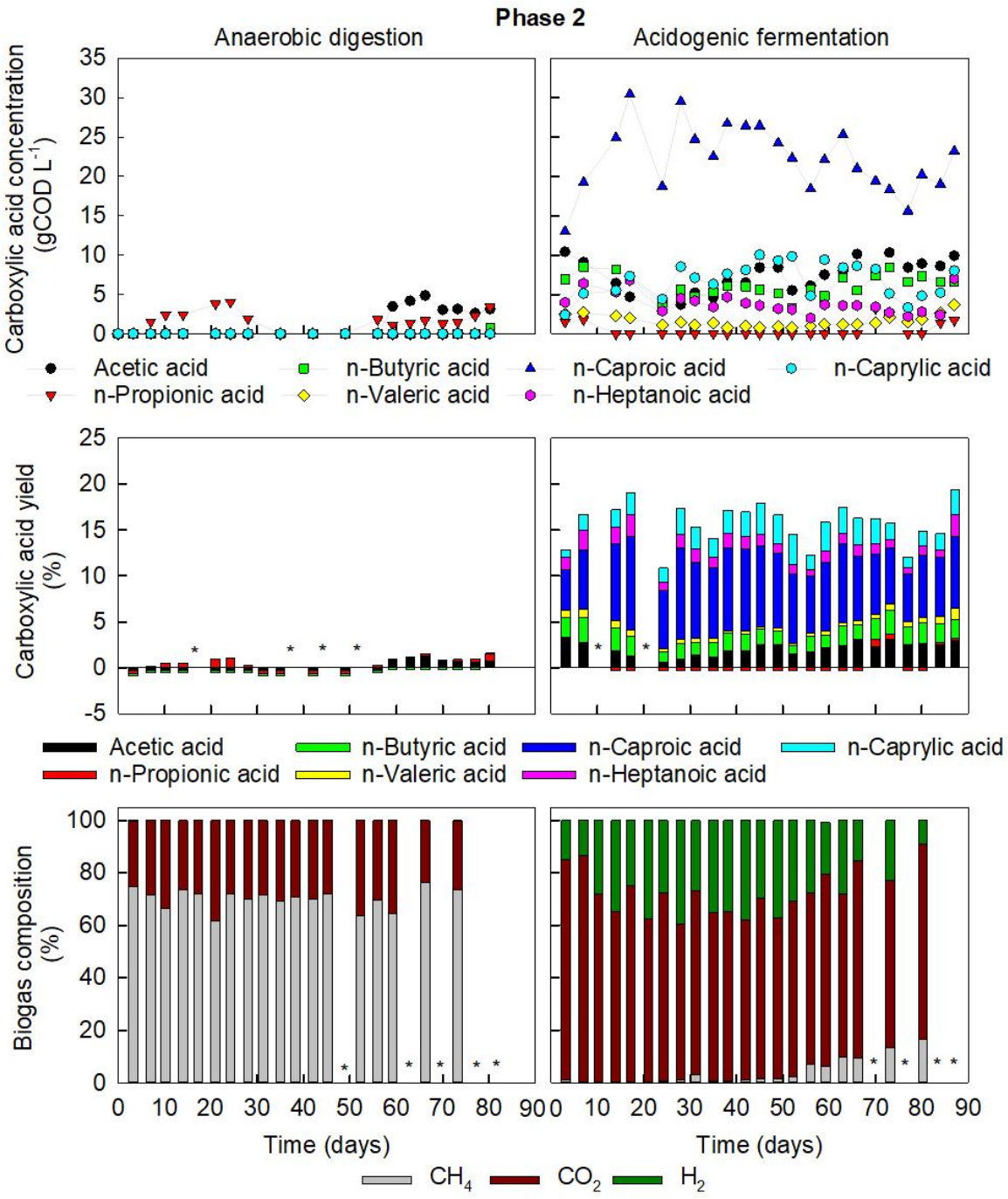


Figure 3-2 *Key chemical compounds in Phase 2 of reactor operation in AD (left) and AF (right). Concentrations (top) and yields (middle) of liquid fermentation compounds and biogas composition (bottom). *Not determined*

In Phase 2, operation of AF was resumed and maintained over five HRT to evaluate the long-term effects of an elevated OLR on product outcome and community enrichment. The average OLR in AF was gradually increased after starvation over 4 feeding events from 9.2 to 21 ± 2 gCOD L^{-1} d^{-1}, slightly higher than at the end of Phase 1. The increase in OLR resulted in an accumulation of carboxylic acids averaging 48 ± 7 gCOD L^{-1}, similar to the end of Phase 1, but with a larger fraction of C5-C8 (73 ± 8 %). On resuming operation, C2 immediately decreased, followed by a decline in C4 four feeding events later (Figure 3-2). The drop in short VFA was accompanied by an increase in C6 and C8, indicating chain elongation. The simple STR setup used in the current study, analogous to current industrial AD setups, resulted in C6 and C8 concentrations of 10.1 ± 1.7g L^{-1} (22.3 ± 3.6 gCOD L^{-1}) and 2.9 ± 0.8 g L^{-1} (7.2 ± 2.0 gCOD L^{-1}), respectively, averaged over five HRT. The maximum concentration of undissociated C6 (with antimicrobial properties) was 2.3 g L^{-1} (Day 17 - Phase 2, pH 5.55, total C6 of 13.8 g L^{-1}). This is far above the reported inhibitory concentration of 0.87 g L^{-1} [53].

By increasing OLR in Phase 2, the loading rate of electron donors, i.e., ethanol and lactic acid present in FW2, slightly rose from 0.25 ± 0.02 gCOD L^{-1} d^{-1} to 0.31 ± 0.03 gCOD L^{-1} d^{-1}. However, the net production rate of MCCA nearly doubled from 1.3 ± 0.1 gCOD L^{-1} d^{-1} to 2.4 ± 0.5 gCOD L^{-1} d^{-1}, so the improved chain elongation could not have been due to electron donors in the influent alone, further indicating stimulation of their *in situ* production by increased OLR.

All carboxylates with an uneven carbon chain length decreased, with C3 dropping below detection levels, followed by a decline in C5 and C7 concentrations (Figure 3-2). C3 production from lactic acid through the acrylate pathway is characterised as a competitive pathway of chain elongation, and occurs at high concentrations of lactic acid and at a pH above 6 [54-56]. It is hypothesised that by operating at a pH between 5.5 and 6.0 in AF, C3 production was minimized due to increased chain elongation consuming lactic acid. The limited presence of C3 resulted in a higher selectivity for MCCA with even numbers of carbon (C6 and C8).

At the start of Phase 2, the F/M in AF operation dropped below 1 gCOD gVS^{-1}, likely due to solid accumulation, yet CH_4 in the biogas remained below 2 % (with the rest being CO_2 and H_2). From Day 56 onwards, the methane fraction increased again and reached a maximum of 17 % on Day 80, despite carboxylic acids and OLR being far above accepted values for inhibition of methanogenesis.

3.1.3.3. Presence of hydrogen and pH stabilisation indicate chain elongation

To gain an insight into the cascade of reactions occurring in AD and AF in between feeding events, gas production and carboxylic acid concentrations were followed by regular sampling between two feeding events (Phase 2, Days 21 to 24) (Figure 3-3). For the AD reactor, the pH profile steadily increased, as often observed with methane production. Only C3 was present in AD, which remained relatively constant (less than 7 % change from time 0). Methane production showed a batch-like production profile with a maximum production rate obtained within the first 24 hours from readily biodegradable matter, followed by a slower production rate as further bioavailable matter was consumed.

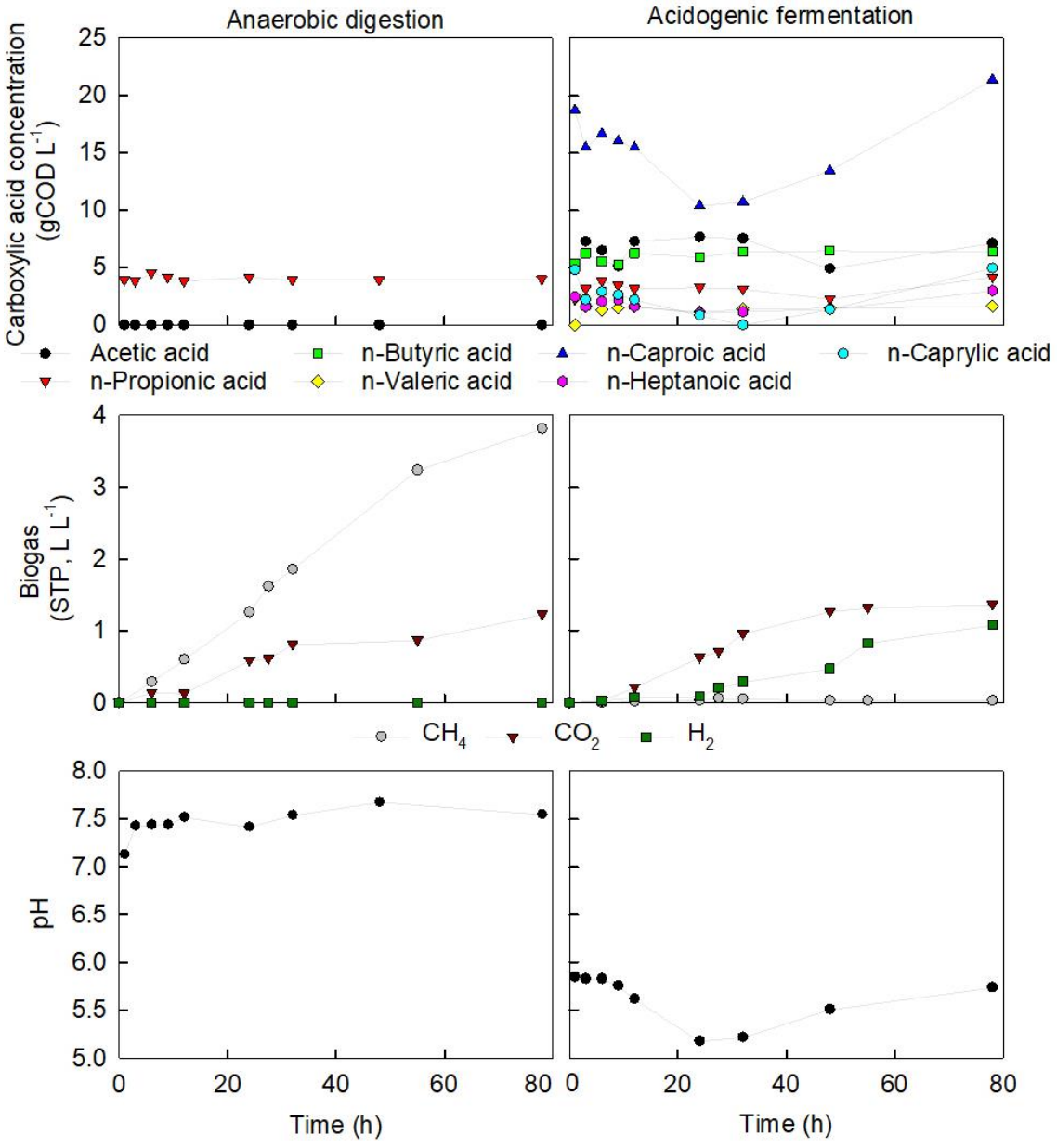


Figure 3-3 *Concentration of carboxylic acids (top), biogas production (middle) and pH profile (bottom) in between two feeding events (Day 21 and 24 of Phase 2) for AD (left) and AF (right). Time 0 corresponds to a sample taken straight after feed addition.*

In the AF reactor, the pH decreased from 5.8 to a minimum of 5.2 during the first 32 hours after feeding. This is in line with primary acidogenic fermentation where short VFA accumulate and acidification occurs. C5-C8 concentrations were nearly halved while C2-C4 increased by 32 %. The reactor headspace had to be opened to introduce feed, thus reducing the pH_2 near 0 atm. An elevated H_2 partial pressure (pH_2), higher than 0.003 atm, has to be maintained to ensure a sufficiently reductive environment and avoid the degradation of MCCA into short VFA via the β-oxidation pathway [53]. In between feeding events, the reactors were kept airtight and after 32 hours of primary fermentation, the pH_2 in AF headspace reached approximately 0.21 atm. The H_2 could have been produced by various metabolic pathways such as primary fermentation, C4 fermentation, acetogenic activity where C2 is converted to CO_2 and H_2 and within the first step in chain elongation, namely ethanol and lactic acid oxidation [4, 14, 57, 58]. In the following 46 hours, pH increased again to 5.7, and H_2 and MCCA increased, indicating a secondary fermentation stage of chain elongation [37]. These consecutive fermentation stages where an initial acidification stage is followed by chain elongation is similar to that reported for other chain elongation studies [37, 57]. It can be hypothesised that this metabolism was the same in our reactor, although we were unable to analyse the lactic acid and ethanol concentrations to confirm it in this case.

3.1.3.4. A distinct enriched microbiome for chain elongation

The effect of reactor operation on the enrichment of the microbial community was evaluated at the end of Phase 2 (Figure 3-4). An average of 159 unique observed operational taxonomic units (OTUs) was found in the AD reactor, whereas only an average of 104 OTUs was observed in AF. Similarly, alpha-diversity, richness and evenness measures were lower for the AF microbiome than for AD (Table 3-3). The AD reactor showed a high relative abundance of Firmicutes (43 ± 2%), Bacteroidetes (18.8 ± 0.1%), Euryarchaeota (10.3 ± 0.1%), and other phyla that are commonly found in anaerobic digesters processing FW (Figure 3-4) [59, 60]. Several hydrolytic and acidogenic groups were detected, such as the proteolytic Firmicutes *Gallicola* (10 ± 2%) and *Fastidiosipila* (12 ± 1%), and the lactic acid-producing *Enterococcus* (1.3 ± 0.3%). The detected bacteria belonging to Bacteroidetes and Synergistetes are generally important hydrolysers in AD that degrade carbohydrates and proteinaceous substrates, and contribute to acidogenesis by producing VFA and other acids [61-64]. This is in line with stabilization of waste streams by reducing the solid and organic content of the feedstock in AD, with an average VS and COD removal of 84 ± 9 % and 83 ± 5 %, respectively, similar to other FW AD studies (42 to 95%) [65]. The various genera that play crucial roles in hydrolysis and acidogenic fermentation present in AD could not be found in in the AF reactor, although some other genera were detected. In the AF

reactor, the VS removal only achieved 36 ± 21% in accordance with a lower hydrolytic community. Meanwhile, COD removal in AF accounted for 28 ± 16%, as most of the organics were retained as VFA instead of degassed via methane, although the relative abundance of the archaeal community was similar to that in AD, as discussed below.

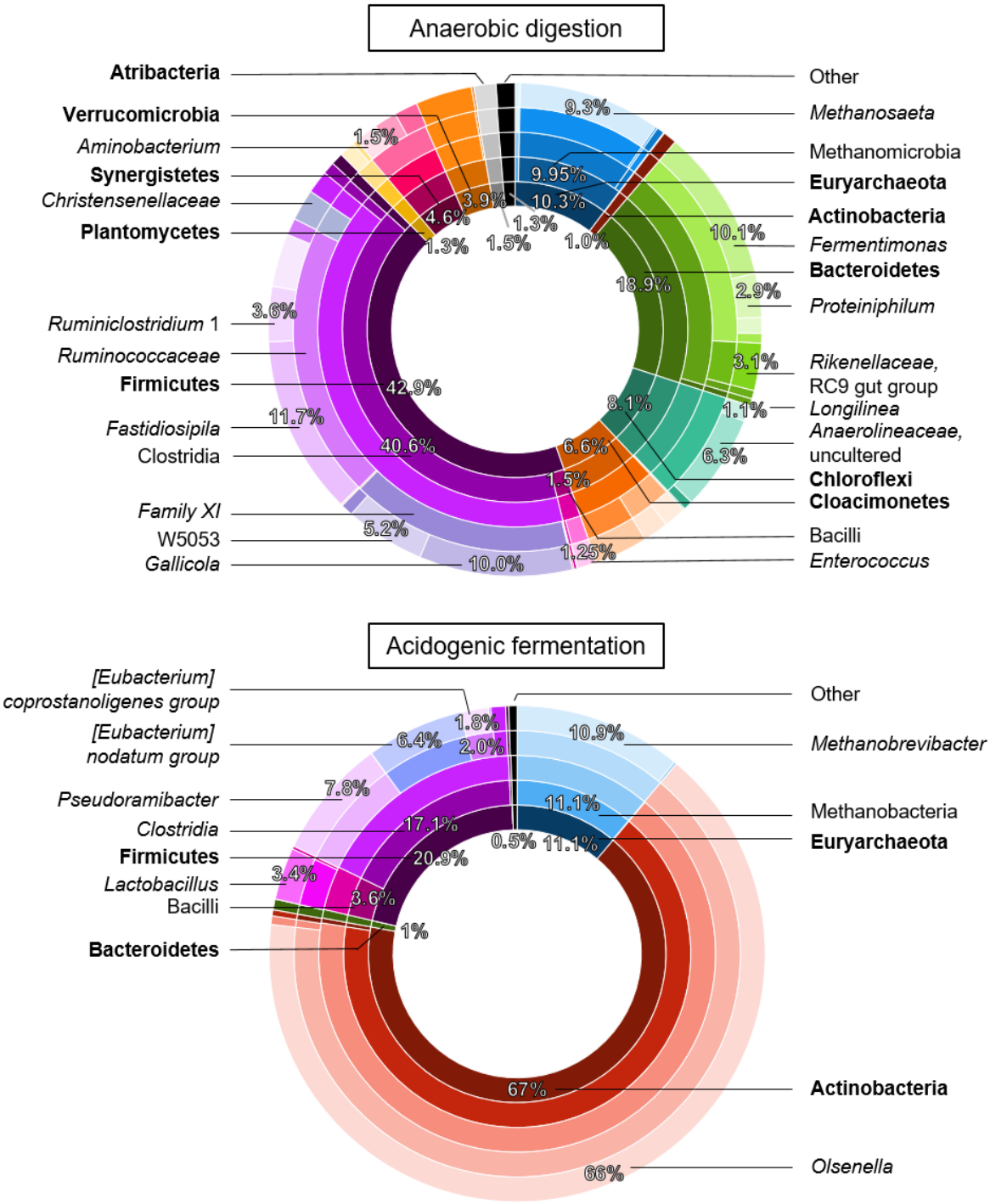


Figure 3-4 *Taxonomic composition of the bacterial and archaeal community by the end of Phase 2: the anaerobic digestion reactor on Day 77 (top) and, the acidogenic fermentation reactor on Day 84 of operation (bottom). The concentric circles represent the taxonomic classification from phylum (bold, centre ring) to genus (outer ring), colours represent classification, and bandwidth and percentages relate to relative abundance.*

Table 3-3 *Alpha-diversity indices for the microbial community in the AD and AF reactors. Averaged over duplicate samples and calculated based on 20,314 reads per sample.*

Index	AD	AF
Observed OTUs	159 ± 4	102 ± 7
Shannon	3.49 ± 0.04	1.70 ± 0.08
Simpson	0.945 ± 0.007	0.66 ± 0.04
InvSimpson	18.6 ± 0.8	2.9 ± 0.3
Chao1 (richness)	182 ± 16	129 ± 5
ACE (richness)	184 ± 18	135 ± 14
Pielou evenness	0.625 ± 0.007	0.31 ± 0.01

The community during AF was dominated by Actinobacteria, comprising of predominantly the genus *Olsenella* (66 ± 4% over 8 OTUs in AF, <0.1% in AD). The acidotolerant *Olsenella* sp. and the highly diverse genus of *Lactobacillus* (3 ± 2% over 13 OTUs in AF, <0.1% in AD) are linked with hydrolysis and the acidogenic production of, for instance, lactic acid, acetate, CO_2, and ethanol from hexoses and pentoses [66-68]. In addition, these genera have been correlated with lactic acid-based chain elongation. *Olsenella* sp. have been found in reactors processing lignocellulosic substrates in co-occurrence with bacteria from the chain elongating genus *Pseudoramibacter*, the third most abundant genus (7.83 ± 0.08%) in our reactor [35, 36, 69]. *Pseudoramibacter* spp. produce VFA, MCCA and H_2 by fermenting carbohydrates, and, as recently suggested, glycerol, and lactic acid [70, 71]. *Lactobacillus* spp. have been detected in FW fermentation alongside *Caproiciproducens* spp., which only had a low relative abundance of 0.53 ± 0.09% in our reactor [37]. Other abundant genera in AF were from the order of Clostridiales. The genus classified as the *Eubacterium nodatum* group (6.4 ± 0.5%) is known for the decomposition of organic matter into VFA and was found before to compete with chain elongation bacteria resulting in excessive C4 production in a xylan-fed fermentation [69]. The genus *E. coprostanoligenes* group (1.8 ± 0.3%) has been found before in FW digesters producing C2, succinic acid and H_2 and has a phospholipase activity to reduce cholesterol [60, 72].

In terms of the archaeal community, the relative abundance of methanogenic Euryarchaeota was similar in the AD and AF reactor (Figure 3-4). In the AD reactor, the dominant genus was the acetoclastic methanogen *Methanoseata* in AD, which is typical for full-scale AD of organic solid waste [73-75]. In contrast, in AF the hydrogenotrophic *Methanobrevibacter* was the lead methanogenic genus. Hydrogenotrophic methanogens have been found to increase in relative abundance during organic overloading of AD systems, and they are generally more tolerant to environments with high carboxylic acids content [8, 76]. This could explain why methane in the AF reactor increased again from Day 56 (Phase 2) onwards, despite the high carboxylic acid concentration.

3.1.4. Discussion

The functionality of the AD seeding sludge shifted to AF when starting reactor operation at a higher F/M and OLR compared to traditional AD. This is consistent with the response of an anaerobic microbiome to a high organic load, whereby accumulation of VFA inhibits methanogenesis [19, 77]. Increased substrate availability by operating at a higher OLR (and indirectly F/M), while maintaining HRT (i.e., increased COD in the FW 2) shifted product outcome further from methane to VFA, and from short VFA to MCCA. Chain elongation improved despite a decreased supply of electrons donors in the influent (i.e., FW2). Normally, the elongation of VFA to MCCA via the reversal of the β-oxidation pathway becomes less thermodynamically favourable with less electron donors available [53, 78]. However, Arslan, et al. [79] reviewed several mixed culture AF studies and showed that an increase in organic load generally resulted in a more reduced product spectrum of carboxylic acid. During FW fermentation, the electron donors for chain elongation can be produced *in situ* [37]. The co-occurrence of lactate producing *Olsenella* spp. and lactate consuming chain elongation bacteria with increased availability of substrates confirms that the AF microbiome was able to produce the electron donors required for chain elongation *in situ*, alongside using the few electron donors in the feedstock.

Increased availability of organics, either by high F/M or OLR, lead to VFA accumulation, which in the case of AD jeopardised the main goal of the process. Doubling the HRT, and thus decreasing OLR, temporarily restored the functionality of the AD reactor. However, in the long-term, working at maximum AD capacity accumulated n-propionic acid, difficult to degrade, which inhibited methanogenesis. Thus, whereas AD requires a more dilute FW feedstock or operation with extended retention times to allow mitigation of OLR stress, high-COD FW streams lend themselves well as feedstock for MCCA production as they allow accumulating electron donors from primary fermentation.

The overall values for C6 are higher than reported for similar un-supplemented STR setups; for instance, 8.5 g L^{-1} C6 were produced with switch-grass stillage feedstock [36]. The concentrations in the current study were closer to those fermenting FW using more sophisticated reactor set-ups, such as a leach bed reactors (9.9 g L^{-1} C6) [31], or a two-stage ethanol-supplemented up-flow anaerobic reactor (12.6 g L^{-1}) [28]. Higher concentration of 23 ± 1 g L^{-1} C6 have been reported but only when chain elongation was further stimulated by using pre-treated FW, ethanol supplementation and a microbiome previously enriched with synthetic media [80]. However, these FW substrates might differ in composition such as solid or COD content, similar, as FW 1 was different from FW 2. For fermentation of acid whey it has recently been found that the quality of the feedstock had

significant impact on MCCA production [81]. Further research should evaluate the impact of FW composition as the application of this technology would have to deal with the inherent variability of a FW substrate caused by differences in sources, collection and storage [82, 83].

Concentrations of C8 have reached higher concentrations in similar reactor set-ups using alternative feedstocks, e.g., 3.2 g L^{-1} fermenting thin stillage and beer [84] and 3.1 ± 0.9 g L^{-1} for diluted cheese whey powder [85]. These specific feedstocks are high in ethanol from beer and lactic acid from whey, creating a more reductive environment that could stimulate further chain elongation. Thus, better operational control to ensure reductive conditions could enhance the elongation process. For instance, an airtight feeding strategy could improve maintaining a sufficient pH_2. Kinetic studies between feed events indicated that C4-C8 compounds were partially degraded right after a feed event, likely due to loss of pH_2. Future work should also evaluate whether application of a semi-continuous feeding pattern instead of continuous, i.e., subjecting the microbiome to a fluctuating substrate availability, maximizes the benefits of consecutive fermentation stages as seen in batch-like operation. Namely, they stimulate the initial, rapid accumulation of electron donors by primary fermentation and, thus, ensure reductive conditions for consecutive chain elongation.

For anaerobic microbiomes, it is generally regarded that in response to operational changes the hydrolytic and acidogenic bacteria are sufficiently dynamic and able to maintain overall functionality by replacing one another, whereas methanogenic activity will subside after operational disturbance and potentially rebound later on [86]. This typical quality of open fermentation systems where multiple distinct microorganisms are capable of performing similar biochemical function, i.e., functional redundancy, is seen as advantageous since it allows to stabilize reactor functionality following operational perturbation, e.g., substrate fluctuations, or adapt to new environmental conditions [87, 88]. Indeed, different genera were responsible for the main functionalities of hydrolysis and acidogenic fermentation in the AD and AF reactors. Thus, these key metabolic functionalities required for dealing with a complex feedstock in AD were maintained in AF. However, the same concept allowed for resilient methanogenic activity by the development of hydrogenotrophic methanogens in the AF reactor. Since a decrease of pH_2 due to hydrogenotrophic methanogenic activity could potentially compromise MCCA yields, the implications for full-scale long-term application should be further evaluated.

The lower pH, i.e., more acid-stress, and shorter retention times in the AF reactor, reduced degradation of the FW solids, as seen by the lower VS removal and reshaped the microbial community of AF into a more specialised and homogenous community than in the AD

reactor. This decrease in community richness, diversity and evenness is in line with what other studies reported for organic overloading of AD and for chain elongation studies producing C8 [76, 84, 89]. Improvements in hydrolysis and MCCA yield, or integration within a broader bio-refinery context that includes post chain elongation treatment will be required in practise if equivalent waste reduction and stabilization to AD are to be obtained.

High investments in highly specific infrastructure and/or lack of skills and expertise are some of the main technical barriers hindering adoption of advanced wastewater treatment technologies [90, 91]. Here we have shown that MCCA production can be stimulated from FW fermentation without supplementation of methanogenic inhibitors, electron donors or growth medium in a simple, single-stage STR by manipulation of the organic load. Thus, it is similar to the current operation of established AD systems, in particular it is comparable to acid-phase digesters that also operate at increased organic load and lower pH. In addition, the high-COD FW substrate that required extended retention times or dilution for AD, is more advantageous to apply as substrate for MCCA production hence overcoming some of the difficulties faced in FW AD. However, while the fermentation process itself could allow repurposing current digesters, significant research efforts are still required regarding separation and purification of MCCA to obtain marketable products.

Acknowledgements and funding

This research has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement H2020-MSCA-CO-FUND, No 665992. We would like to thank the support and funding from Wessex Water and GENeco (Bristol, UK). This work is supported by EPSRC under grant EP/L016354/1. The authors would like to thank Tom Phelps, Ian Law and Wesley Wong from GENeco for reliable and enthusiastic support of the research performed in the supply of samples and information.

References

[1] D. A. Teigiserova, L. Hamelin, and M. Thomsen, "Review of high-value food waste and food residues biorefineries with focus on unavoidable wastes from processing," *Resources, Conservation and Recycling,* vol. 149, pp. 413-426, 2019.

[2] Å. Stenmarck, C. Jensen, T. Quested, and G. Moates, "Estimates of European food waste levels," Fusions EU, Stockholm978-91-88319-01-2, 2016.

[3] F. Xu, Y. Li, X. Ge, L. Yang, and Y. Li, "Anaerobic digestion of food waste - Challenges and opportunities," *Bioresour Technol,* vol. 247, pp. 1047-1058, Jan 2018.

[4] M. T. Agler, B. A. Wrenn, S. H. Zinder, and L. T. Angenent, "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform," *Trends Biotechnol,* vol. 29, no. 2, pp. 70-8, Feb 2011.

[5] C. Urban *et al.*, "Production of drop-in fuels from biomass at high selectivity by combined microbial and electrochemical conversion," *Energy & Environmental Science,* vol. 10, no. 10, pp. 2231-2244, 2017.
[6] V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, "Medium Chain Carboxylic Acids from Complex Organic Feedstocks by Mixed Culture Fermentation," *Molecules,* vol. 24, no. 3, Jan 22 2019.
[7] W. Han, P. He, L. Shao, and F. Lü, "Road to full bioconversion of biowaste to biochemicals centering on chain elongation: A mini review," *Journal of Environmental Sciences,* vol. 86, pp. 50-64, 2019.
[8] I. Vanwonterghem, P. D. Jensen, K. Rabaey, and G. W. Tyson, "Temperature and solids retention time control microbial population dynamics and volatile fatty acid production in replicated anaerobic digesters," *Sci Rep,* vol. 5, p. 8496, Feb 16 2015.
[9] S. Greses, E. Tomás-Pejó, and C. González-Fernández, "Short-chain fatty acids and hydrogen production in one single anaerobic fermentation stage using carbohydrate-rich food waste," *Journal of Cleaner Production,* 2020.
[10] M. S. Kim *et al.*, "More value from food waste: Lactic acid and biogas recovery," *Water Res,* vol. 96, pp. 208-16, Jun 1 2016.
[11] X. Xu *et al.*, "Stabilizing lactate production through repeated batch fermentation of food waste and waste activated sludge," *Bioresour Technol,* vol. 300, p. 122709, Mar 2020.
[12] C. Cavinato, A. Giuliano, D. Bolzonella, P. Pavan, and F. Cecchi, "Bio-hythane production from food waste by dark fermentation coupled with anaerobic digestion process: A long-term pilot scale experience," *International Journal of Hydrogen Energy,* vol. 37, no. 15, pp. 11549-11555, 2012.
[13] S. Im, M.-K. Lee, Y.-M. Yun, S.-K. Cho, and D.-H. Kim, "Effect of storage time and temperature on hydrogen fermentation of food waste," *International Journal of Hydrogen Energy,* vol. 45, no. 6, pp. 3769-3775, 2020.
[14] C. M. Spirito, H. Richter, K. Rabaey, A. J. Stams, and L. T. Angenent, "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr Opin Biotechnol,* vol. 27, pp. 115-22, Jun 2014.
[15] P. J. Weimer and R. A. Kohn, "Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside?," *Appl Microbiol Biotechnol,* vol. 100, no. 8, pp. 3389-98, Apr 2016.
[16] W. S. Chen, D. Strik, C. J. N. Buisman, and C. Kroeze, "Production of Caproic Acid from Mixed Organic Waste: An Environmental Life Cycle Perspective," *Environ Sci Technol,* vol. 51, no. 12, pp. 7159-7168, Jun 20 2017.
[17] D. Arslan, K. J. Steinbusch, L. Diels, H. De Wever, H. V. Hamelers, and C. J. Buisman, "Selective carboxylate production by controlling hydrogen, carbon dioxide and substrate concentrations in mixed culture fermentation," *Bioresour Technol,* vol. 136, pp. 452-60, May 2013.
[18] K. J. Steinbusch, E. Arvaniti, H. V. Hamelers, and C. J. Buisman, "Selective inhibition of methanogenesis to enhance ethanol and n-butyrate production through acetate reduction in mixed culture fermentation," *Bioresour Technol,* vol. 100, no. 13, pp. 3261-7, Jul 2009.
[19] W. Zhang, W. Xing, and R. Li, "Real-time recovery strategies for volatile fatty acid-inhibited anaerobic digestion of food waste for methane production," *Bioresource Technology,* vol. 265, pp. 82-92, Oct 2018.
[20] M. Coma *et al.*, "Organic waste as a sustainable feedstock for platform chemicals," *Faraday Discuss,* vol. 202, pp. 175-195, Sep 21 2017.
[21] L. R. Jarboe, L. A. Royce, and P. Liu, "Understanding biocatalyst inhibition by carboxylic acids," *Front Microbiol,* vol. 4, p. 272, Sep 3 2013.
[22] X. Zhu *et al.*, "The synthesis of n-caproate from lactate: a new efficient process for medium-chain carboxylates production," *Sci Rep,* vol. 5, p. 14360, Sep 25 2015.

[23] L. A. Kucek, J. Xu, M. Nguyen, and L. T. Angenent, "Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction," *Front Microbiol,* vol. 7, p. 1892, 2016.
[24] S. J. Andersen *et al.*, "Electrolytic extraction drives volatile fatty acid chain elongation through lactic acid and replaces chemical pH control in thin stillage fermentation," *Biotechnol Biofuels,* vol. 8, p. 221, 2015.
[25] A. Duber *et al.*, "Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey," *Green Chemistry,* vol. 20, no. 16, pp. 3790-3803, 2018.
[26] J. M. Carvajal-Arroyo *et al.*, "Granular fermentation enables high rate caproic acid production from solid-free thin stillage," *Green Chemistry,* vol. 21, no. 6, pp. 1330-1339, 2019.
[27] Q. Wu, X. Feng, W. Guo, X. Bao, and N. Ren, "Long-term medium chain carboxylic acids production from liquor-making wastewater: Parameters optimization and toxicity mitigation," *Chemical Engineering Journal,* vol. 388, 2020.
[28] T. I. M. Grootscholten, D. P. B. T. B. Strik, K. J. J. Steinbusch, C. J. N. Buisman, and H. V. M. Hamelers, "Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol," *Applied Energy,* vol. 116, pp. 223-229, 2014.
[29] J. Xu, J. Hao, J. J. L. Guzman, C. M. Spirito, L. A. Harroff, and L. T. Angenent, "Temperature-Phased Conversion of Acid Whey Waste Into Medium-Chain Carboxylic Acids via Lactic Acid: No External e-Donor," *Joule,* vol. 2, no. 2, pp. 280-295, 2018.
[30] W. C. Khor, S. Andersen, H. Vervaeren, and K. Rabaey, "Electricity-assisted production of caproic acid from grass," *Biotechnol Biofuels,* vol. 10, p. 180, 2017.
[31] C. O. Nzeteu, A. C. Trego, F. Abram, and V. O'Flaherty, "Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system," *Biotechnol Biofuels,* vol. 11, p. 108, 2018.
[32] H. Ma *et al.*, "Effect of ultrasonic pretreatment on chain elongation of saccharified residue from food waste by anaerobic fermentation," *Environmental Pollution,* 2020.
[33] N. Bachmann, "Design and engineering of biogas plants," in *Biogas Handbook: Science, Production and Applications*, vol. 52, A. Wellinger, J. D. Murphy, D. Baxter, and R. Braun, Eds. no. Woodhead Publishing Series in Energy Ser.): Elsevier Science & Technology, 2013, pp. 191-211.
[34] J. D. Murphy and T. Thamsiriroj, "Fundametal science and engineering of the anaerobic digestion process for biogas production," in *Biogas Handbook: Science, Production and Application*, vol. 52, A. Wellinger, J. D. Murphy, D. Baxter, and R. Braun, Eds.: Elsevier Science & Technology, 2013.
[35] J. Lambrecht *et al.*, "Key sub-community dynamics of medium-chain carboxylate production," *Microb Cell Fact,* vol. 18, no. 1, p. 92, May 28 2019.
[36] M. J. Scarborough, G. Lynch, M. Dickson, M. McGee, T. J. Donohue, and D. R. Noguera, "Increasing the economic value of lignocellulosic stillage through medium-chain fatty acid production," *Biotechnol Biofuels,* vol. 11, p. 200, 2018.
[37] C. A. Contreras-Davila, V. J. Carrion, V. R. Vonk, C. N. J. Buisman, and D. Strik, "Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation," *Water Res,* vol. 169, p. 115215, Feb 1 2020.
[38] APHA, "Standard methods for the examination of water and wastewater," vol. 23, 2017.
[39] G. Manni and F. Caron, "Calibration and determination of volatile fatty acids in waste leachates by gas chromatography," *Journal of Chromatography A,* vol. 690, pp. 237-242, 1995.
[40] M. Albertsen, S. M. Karst, A. S. Ziegler, R. H. Kirkegaard, and P. H. Nielsen, "Back to Basics-The Influence of DNA Extraction and Primer Choice on Phylogenetic

Analysis of Activated Sludge Communities," *PLoS One,* vol. 10, no. 7, p. e0132783, 2015.
[41] I. Illumina, "16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B," 2015.
[42] A. Apprill, S. McNally, R. Parsons, and L. Weber, "Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton," *Aquatic Microbial Ecology,* vol. 75, no. 2, pp. 129-137, 2015.
[43] A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: a flexible trimmer for Illumina sequence data," *Bioinformatics,* vol. 30, no. 15, pp. 2114-20, Aug 1 2014.
[44] R. C. Edgar, "UPARSE: highly accurate OTU sequences from microbial amplicon reads," *Nat Methods,* vol. 10, no. 10, pp. 996-8, Oct 2013.
[45] T. Magoc and S. L. Salzberg, "FLASH: fast length adjustment of short reads to improve genome assemblies," *Bioinformatics,* vol. 27, no. 21, pp. 2957-63, Nov 1 2011.
[46] J. G. Caporaso *et al.*, "QIIME allows analysis of high-throughput community sequencing data," *Nat Methods,* vol. 7, no. 5, pp. 335-6, May 2010.
[47] C. Quast *et al.*, "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools," *Nucleic Acids Res,* vol. 41, no. Database issue, pp. D590-6, Jan 2013.
[48] Q. Wang, G. M. Garrity, J. M. Tiedje, and J. R. Cole, "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy," *Appl Environ Microbiol,* vol. 73, no. 16, pp. 5261-7, Aug 2007.
[49] R Core Team, "R: A language and envionment for statistical computing," ed. Vienna, Austria: R Foundation for Statistical Computing, 2017.
[50] V. De Groof, M. Coma Bech, D. J. Leak, T. Arnot, and A. Lanham, "Dataset for "Adjusting organic load as a strategy to direct single-stage food waste fermentation from anaerobic digestion to chain elongation"," ed. Bath: University of Bath Research Data Archive, 2020.
[51] I. Siegert and C. Banks, "The effect of volatile fatty acid additions on the anaerobic digestion of cellulose and glucose in batch reactors," *Process Biochemistry,* vol. 40, no. 11, pp. 3412-3418, 2005.
[52] M. A. Voelklein, R. O' Shea, A. Jacob, and J. D. Murphy, "Role of trace elements in single and two-stage digestion of food waste at high organic loading rates," *Energy,* vol. 121, pp. 185-192, 2017.
[53] L. T. Angenent *et al.*, "Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals," *Environ Sci Technol,* vol. 50, no. 6, pp. 2796-810, Mar 15 2016.
[54] R. Prabhu, E. Altman, and M. A. Eiteman, "Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions," *Appl Environ Microbiol,* vol. 78, no. 24, pp. 8564-70, Dec 2012.
[55] L. A. Kucek, M. Nguyen, and L. T. Angenent, "Conversion of L-lactate into n-caproate by a continuously fed reactor microbiome," *Water Res,* vol. 93, pp. 163-171, Apr 15 2016.
[56] P. Candry, L. Radic, J. Favere, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Mildly acidic pH selects for chain elongation to caproic acid over alternative pathways during lactic acid fermentation," *Water Res,* vol. 186, p. 116396, Sep 7 2020.
[57] H. Sträuber, M. Schröder, and S. Sabine Kleinsteuber, "Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process," *Energy, Sustainability and Society,* vol. 2, no. 13, 2012.
[58] N. H. Yasin, T. Mumtaz, M. A. Hassan, and N. Abd Rahman, "Food waste and food processing waste for biohydrogen production: a review," *J Environ Manage,* vol. 130, pp. 375-85, Nov 30 2013.
[59] J. Cardinali-Rezende, P. Rojas-Ojeda, A. M. Nascimento, and J. L. Sanz, "Proteolytic bacterial dominance in a full-scale municipal solid waste anaerobic

reactor assessed by 454 pyrosequencing technology," *Chemosphere,* vol. 146, pp. 519-25, Mar 2016.

[60] X. Peng, S. Zhang, L. Li, X. Zhao, Y. Ma, and D. Shi, "Long-term high-solids anaerobic digestion of food waste: Effects of ammonia on process performance and microbial community," *Bioresour Technol,* vol. 262, pp. 148-158, Aug 2018.

[61] S. Hahnke, T. Langer, D. E. Koeck, and M. Klocke, "Description of Proteiniphilum saccharofermentans sp. nov., Petrimonas mucosa sp. nov. and Fermentimonas caenicola gen. nov., sp. nov., isolated from mesophilic laboratory-scale biogas reactors, and emended description of the genus Proteiniphilum," *Int J Syst Evol Microbiol,* vol. 66, no. 3, pp. 1466-1475, Mar 2016.

[62] N. R. Krieg, W. Ludwig, J. Euzéby, and W. B. Whitman, "Phylum XIV. Bacteroidetes phyl. nov.," in *Bergey's Manual® of Systematic Bacteriology*, N. R. Krieg *et al.*, Eds. New York: Springer, 2010.

[63] S. Baena *et al.*, "Aminobacterium colobiense gen. nov. sp. nov., an amino acid-degrading anaerobe isolated from anaerobic sludge," *Anaerobe,* vol. 4, pp. 241-250, 1998.

[64] M. C. Nelson, M. Morrison, and Z. Yu, "A meta-analysis of the microbial diversity observed in anaerobic digesters," *Bioresour Technol,* vol. 102, no. 4, pp. 3730-9, Feb 2011.

[65] C. M. Braguglia, A. Gallipoli, A. Gianico, and P. Pagliaccia, "Anaerobic bioconversion of food waste into energy: A critical review," *Bioresour Technol,* vol. 248, no. Pt A, pp. 37-56, Jan 2018.

[66] F. E. Dewhirst *et al.*, "Characterization of novel human oral isolates and closed 16S rDNA sequences that fall in the family coriobacteriaceae: description of Olsenella gen. nov., reclassification of Lactobacillus ulo as Olsenella uli comb. nov. and description of Olsenella profusa sp. nov.," *International journal of Systematic and Evolutionary Microbiology,* vol. 51, pp. 1797-1804, 2001.

[67] J. Zheng *et al.*, "A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae," *Int J Syst Evol Microbiol,* Apr 15 2020.

[68] M. Kraatz, R. J. Wallace, and L. Svensson, "Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of Olsenella, Olsenella uli and Olsenella profusa," *Int J Syst Evol Microbiol,* vol. 61, no. Pt 4, pp. 795-803, Apr 2011.

[69] B. Liu, S. Kleinsteuber, F. Centler, H. Harms, and H. Strauber, "Competition Between Butyrate Fermenters and Chain-Elongating Bacteria Limits the Efficiency of Medium-Chain Carboxylate Production," *Front Microbiol,* vol. 11, p. 336, 2020.

[70] M. J. Scarborough, K. S. Myers, T. J. Donohue, and D. R. Noguera, "Medium-Chain Fatty Acid Synthesis by "Candidatus Weimeria bifida" gen. nov., sp. nov., and "Candidatus Pseudoramibacter fermentans" sp. nov," *Appl Environ Microbiol,* vol. 86, no. 3, Jan 21 2020.

[71] A. Willems and M. D. Collins, "Genus VI. Pseudoramibacter," in *Bergey's manual ® of Systematic Bacteriology: The Firmicutes*, vol. 3, P. De Vos *et al.*, Eds. 2 ed.: Springer, 1996, pp. 902-909.

[72] T. A. Freier, D. C. Beitz, L. Li, and P. A. Hartman, "Characterization of Eubacterium coprostanoligenes sp. nov., a cholesterol-reducing anaerobe," *International Journal of Systematic Bacteriology,* vol. 44, no. 1, pp. 137-142, 1994.

[73] Q. Niu, T. Kobayashi, Y. Takemura, K. Kubota, and Y. Y. Li, "Evaluation of functional microbial community's difference in full-scale and lab-scale anaerobic digesters feeding with different organic solid waste: Effects of substrate and operation factors," *Bioresour Technol,* vol. 193, pp. 110-8, Oct 2015.

[74] L. Regueiro *et al.*, "Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters," *Microbiol Res,* vol. 167, no. 10, pp. 581-9, Dec 20 2012.

[75] J. T. Qiao, Y. L. Qiu, X. Z. Yuan, X. S. Shi, X. H. Xu, and R. B. Guo, "Molecular characterization of bacterial and archaeal communities in a full-scale anaerobic reactor treating corn straw," *Bioresour Technol,* vol. 143, pp. 512-8, Sep 2013.
[76] H. M. Jang, J. H. Kim, J. H. Ha, and J. M. Park, "Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater," *Bioresour Technol,* vol. 165, pp. 174-82, Aug 2014.
[77] L. Li, Q. He, Y. Wei, Q. He, and X. Peng, "Early warning indicators for monitoring the process failure of anaerobic digestion system of food waste," *Bioresour Technol,* vol. 171, pp. 491-4, Nov 2014.
[78] W. d. A. Cavalcante, R. C. Leitão, T. A. Gehring, L. T. Angenent, and S. T. Santaella, "Anaerobic fermentation for n-caproic acid production: A review," *Process Biochemistry,* vol. 54, pp. 106-119, 2017.
[79] D. Arslan *et al.*, "Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations," *Critical Reviews in Environmental Science and Technology,* vol. 46, no. 6, pp. 592-634, 2016.
[80] M. Roghair, Y. Liu, D. Strik, R. A. Weusthuis, M. E. Bruins, and C. J. N. Buisman, "Development of an Effective Chain Elongation Process From Acidified Food Waste and Ethanol Into n-Caproate," *Front Bioeng Biotechnol,* vol. 6, p. 50, 2018.
[81] A. Duber, R. Zagrodnik, J. Chwialkowska, W. Juzwa, and P. Oleskowicz-Popiel, "Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation," *Sci Total Environ,* vol. 728, p. 138814, Aug 1 2020.
[82] S. E. Daly, J. G. Usack, L. A. Harroff, J. G. Booth, M. P. Keleman, and L. T. Angenent, "Systematic Analysis of Factors That Affect Food-Waste Storage: Toward Maximizing Lactate Accumulation for Resource Recovery," *ACS Sustainable Chemistry & Engineering,* vol. 8, no. 37, pp. 13934-13944, 2020.
[83] H. Fisgativa, A. Tremier, and P. Dabert, "Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion," *Waste Manag,* vol. 50, pp. 264-74, Apr 2016.
[84] S. J. Andersen *et al.*, "A Clostridium Group IV Species Dominates and Suppresses a Mixed Culture Fermentation by Tolerance to Medium Chain Fatty Acids Products," *Front Bioeng Biotechnol,* vol. 5, p. 8, 2017.
[85] J. M. B. Domingos *et al.*, "Cheese whey integrated valorisation: Production, concentration and exploitation of carboxylic acids for the production of polyhydroxyalkanoates by a fed-batch culture," *Chemical Engineering Journal,* vol. 336, pp. 47-53, 2018.
[86] L. Li, X. Peng, X. Wang, and D. Wu, "Anaerobic digestion of food waste: A review focusing on process stability," *Bioresour Technol,* vol. 248, no. Pt A, pp. 20-28, Jan 2018.
[87] S. Louca *et al.*, "Function and functional redundancy in microbial systems," *Nat Ecol Evol,* vol. 2, no. 6, pp. 936-943, Jun 2018.
[88] C. W. Marshall, E. V. LaBelle, and H. D. May, "Production of fuels and chemicals from waste by microbiomes," *Curr Opin Biotechnol,* vol. 24, no. 3, pp. 391-7, Jun 2013.
[89] M. T. Agler, C. M. Spirito, J. G. Usack, J. J. Werner, and L. T. Angenent, "Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates," *Energy & Environmental Science,* vol. 5, no. 8, 2012.
[90] H. Gupta and M. K. Barua, "A framework to overcome barriers to green innovation in SMEs using BWM and Fuzzy TOPSIS," *Sci Total Environ,* vol. 633, pp. 122-139, Aug 15 2018.
[91] P. Garrone, L. Grilli, A. Groppi, and R. Marzano, "Barriers and drivers in the adoption of advanced wastewater treatment technologies: a comparative analysis of Italian utilities," *Journal of Cleaner Production,* vol. 171, pp. S69-S78, 2018.

Chapter 4. Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters

This chapter tackled the second research objective concluded from the literature review: evaluating how HRT and OLR affect food waste fermentation and MMC composition. In doing this, it build further on identifying operational strategies to produce MCCA from food waste. The mechanisms underlying fermentation were analysed by microbial community analysis and cycle studies, thus, tackling research objectives five and six.

It was critical to evaluate how the interplay between HRT and OLR affect fermentation outcome and the microbial community. Namely, the previous chapter demonstrated that an increased OLR due to an increase in COD-content of the feedstock, stimulated chain elongation (Chapter 3). The effect of HRT was unaddressed. As outlined in the literature review (Chapter 2), the HRT dictates the time available for feedstock hydrolysis and microbial growth. Since the HRT and OLR are inversely related in the stirred tank reactors used, i.e., the reactor setup determined by the thesis scope, it is crucial to understand the implications when trying to target MCCA by increasing OLR, and inevitably decreasing HRT when the COD-content of the feedstock is fixed.

Three combinations of HRT and OLR were tested and three different fermentation profiles were obtained. These results highlighted the product flexibility of acidogenic MMC fermentation. An adaptable product portfolio is attractive for bio-waste valorisation. Therefore, the results were discussed in this context and submitted as a manuscript for publication to the journal Waste Management.

This chapter is submitted in an alternative thesis format in line with Appendix 6A of the “Specifications for Higher Degree Theses and Portfolios” as required by the University of Bath. This work was submitted for publication in Waste Management:

V. De Groof, M. Coma, T. Arnot, D. J. Leak, and A. B. Lanham, “Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters,” *Waste Management*, **Submitted manuscript**

The dataset was made available as:

V. De Groof, M. Coma Bech, T. Arnot, D. J. Leak, and A. Lanham, "Dataset for "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters"," ed. Bath: University of Bath Research Data Archive, 2020. doi:10.15125/BATH-00946

This declaration concerns the article entitled:			
Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters			
Publication status (tick one)			
~~**Draft manuscript**~~	~~**Submitted**~~	**In review**	~~**Accepted**~~ ~~**Published**~~
Publication details (reference)	V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, " Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters"		
Copyright status (tick the appropriate statement)			
I hold the copyright for this material	~~Copyright is retained by the publisher, but I have been given permission to replicate the material here~~		
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: The candidate predominantly formulated the ideas. These were further developed in discussion with and with input from co-authors - 80% Design of methodology: The candidate predominantly designed the methodology. Methodology was optimized and developed further in discussion with co-authors – 80% Experimental work: The candidate predominantly executed the experimental work and data curation. – 100% Presentation of data in journal format: The candidate wrote the original draft of the paper. Co-authors reviewed and proposed edits. – 70%		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	Vicky De Groof	**Date**	27th March 2021

4.1. Submitted research article: Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters

Vicky De Groof[1,2], Marta Coma[3], Tom Arnot[2,3,4], David J Leak[3,4,5], Ana B Lanham[2,4]*

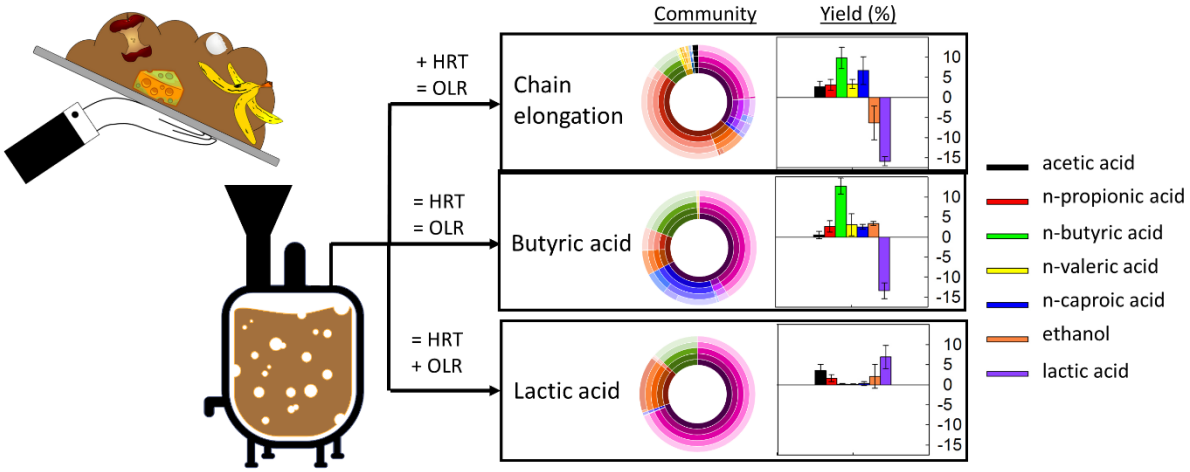
[1] EPSRC Centre for Doctoral Training in Sustainable Chemical Technologies, University of Bath, Claverton Down, Bath BA2 7AY, UK
[2] Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK
[3] Centre for Sustainable and Circular Technologies (CSCT), University of Bath, Claverton Down, Bath BA2 7AY, UK
[4] Water Innovation & Research Centre (WIRC), University of Bath, Claverton Down, Bath BA2 7AY, UK
[5] Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK
* Correspondence: A.Lanham@bath.ac.uk ; Tel.: +441225384544

Submitted: December 2020

Abstract

Acidogenic fermentation is an attractive emerging food waste valorisation technology because a range of value-added products can be obtained. However, to improve product selectivity, a better understanding is required on how operating strategies impact the microbial community and fermentation outcome. This study demonstrated that selection of the hydraulic retention time (HRT) and the organic loading rate (OLR) allowed targeting different fermentation pathways in a single-stage, semi-continuous stirred tank reactor. Three combinations of HRT and OLR were tested in duplicate reactors to distinguish the effect of each operating parameter. Three fermentation profiles with distinct microbial communities were obtained. Predominantly n-butyric acid (13 ± 2 gCOD L^{-1}, 55 ± 14% of carboxylates) was produced when operating at an HRT of 8.5 days and OLR around 12 gCOD $L^{-1}d^{-1}$. Operating at an HRT that was two days longer, yet with similar OLR, stimulated chain elongation (up to 13.6 gCOD L^{-1} of n-caproic acid). This was reflected by a more diverse microbial community with a higher relative abundance of genera related to secondary fermentation such as chain elongation. Operating at a higher OLR (20 gCOD $L^{-1}d^{-1}$) but HRT of 8.5 days, resulted in typical lactic acid fermentation (34 ± 5 gCOD L^{-1}) harbouring a community richer in acid-resistant homofermentative *Lactobacillus* spp. Thus, different fermentation products were obtained in the same reactor configuration by small adjustments in two key operating conditions. These findings demonstrate that a flexible product portfolio can be achieved which improves the economic potential of acidogenic fermentation as food waste valorisation technology.

Graphical abstract



Keywords

Acidogenic fermentation

Butyric acid

Caproic acid

Food waste valorisation

Lactic acid

Resource recovery

4.1.1. Introduction

Effective food waste (FW) management systems should provide cost-efficient resource and energy recovery to enable a circular bio-economy and reach international sustainability targets [1, 2]. However, landfilling and incineration are still widely used for FW disposal, which lead to greenhouse gas emissions, are energy intensive and result in an irrevocable loss of resources [3, 4]. To attain the full material and economic potential of FW valorisation, recycling systems that allow for an assortment of products are key [5, 6].

A range of promising technologies are emerging that use acidogenic fermentation with anaerobic microbial communities to generate different value-added products from bio-wastes, often alternative or supplementary to more conventional anaerobic digestion (AD) [7, 8]. For instance, carbohydrates in FW can be fermented to produce lactic acid, ethanol, volatile fatty acids (carboxylic acids with 1 to 4 carbon atoms, VFA) or hydrogen [9-11]. Consecutive fermentation steps can occur, for instance, lactic acid can be further metabolised to VFA, or VFA can be elongated with lactic acid or ethanol as electron donor to generate medium chain carboxylic acids (5-8 carbon atoms, MCCA) [12, 13]. These products can be extracted to serve as renewable commodity chemicals or liquid fuels [14-16]. Alternatively, the enriched fermentation effluent could serve as feedstock for other biological processes, e.g., polyhydroxyalkanoate (PHA) production, a biodegradable substitute to fossil fuel plastics [17].

However, microbial communities in acidogenic fermentation have a wide and complex metabolic capacity, which makes it challenging to steer acidogenic fermentation selectively towards a target product [18]. Generally, specific fermentation products are targeted in studies by manipulating operating conditions, such as the operating temperature, pH, applied organic loading rate (OLR) and retention times [19]. However, when it comes to industrial-scale operation as currently applied in AD, control is most commonly exerted by manipulating the feeding rate, which determines retention time and OLR [20]. Continuous stirred tank reactors (CSTRs) are the most common reactor configuration for AD on commercial scale due to their ease of operation and low investment and operating costs [21]. Being able to select for target acidogenic fermentation products within a CSTR configuration by adjusting the HRT or OLR, thus similar as current AD operation, would be very attractive as supplementary FW valorisation technology.

Retention time can be defined as (i) in relation to the liquid, i.e., the hydraulic retention time (HRT); or (ii) the solid feedstock fraction and microorganisms, i.e., the sludge retention time (SRT). The SRT drives the composition of the microbial community via differential growth rates between species. For instance, due to the slow growth rate of methanogens, AD must

operate at long SRT values. For the same reason, acidogenic fermentation can be selected instead of methanogenesis by reducing the SRT to less than 5-8 days [22, 23]. The HRT dictates the time available for the feedstock to be hydrolysed and fermented, and hence overall throughput. For complex organic feedstocks, acidification generally improves by operating at retention times of 15 days or more [24, 25]. In addition, increased HRT generally results in a shift in the product spectrum towards more reduced compounds, i.e., longer carboxylic acids [18].

A combination of the HRT and the organic content of the feedstock will determine the organic loading rate (OLR) and the feed-to-microbial ratio (F/M) exerted [18]. Raising the OLR to increase F/M is a strategy applied to target carboxylic acid production by inhibiting methanogenesis via organic overload [26]. Extensive literature reviews show that an increase in OLR generally increases carboxylic acid production, up to a peak between 50 to 100 gCOD $L^{-1}d^{-1}$, which then plateaus or declines [27]. In addition, operating at increased OLR can be used as strategy to shift AD of FW towards VFA accumulation and further to stimulate chain elongation for MCCA production [28]. However, other studies have found that increasing the OLR in acidogenic FW fermentation systems stimulated lactic acid production rather than carboxylic acids [29, 30].

Thus, depending on the study, either VFA, lactic acid or MCCA were targeted by manipulating OLR or retention times. However, in CSTRs, the SRT equals the HRT, and the OLR is inversely related to the HRT. Thus, the selection of an optimal HRT or SRT to target a specific product will affect which OLR can be applied with a particular feedstock and vice versa, which is often overlooked. In addition, limited studies provide a systematic investigation of the impact of these operating parameters on the microbial community. Improved understanding is required on how the OLR and HRT each direct fermentation pathways so that an operating strategy can be designed to target each specific product in acidogenic FW fermentation in CSTRs. This would allow developing manageable operating strategies similar as currently applied in AD that provide a broad and flexible product portfolio for FW valorisation. Therefore, this study aimed at untangling the effect of OLR and HRT on acidogenic fermentation in a semi-continuous STR system. To observe the impact of HRT and OLR on product outcome and on the respective microbial community, duplicate semi-continuous STRs were operated at three different conditions: high HRT with low OLR (HH/LO), low HRT with low OLR (LH/LO), and low HRT with high OLR (LH/HO). These results provide a concrete strategy to direct acidogenic fermentation of FW into different and specific products of interest, and thus, demonstrate the opportunity of repurposing existing single stage AD assets to diversify the product range from FW valorisation.

4.1.2. Materials and Methods

4.1.2.1. Feedstock and inoculum

The feedstock comprised unpackaged Category 3 mixed FW from a full-scale industrial digestion plant (GENeco, Bristol, UK). The FW is a slurry-like mixture obtained in the plant by grounding FW collections from households, restaurants, supermarkets and other catering services, with liquid streams from the food-processing industry and/or the liquid fraction of AD effluent. Upon collection, the FW was stored in aliquots for feeding purposes at -18 °C. Two batches of FW were collected, and each set of duplicate semi-continuous reactors were operated with the same feed throughout (Table 4-1). The fermentation runs HH/LO and LH/LO were inoculated with an acidogenic fermentation culture from an in-house reactor that operated under similar conditions, which was stored at 4°C [28]. The LH/HO systems were inoculated with effluent from HH/LO reactors.

Table 4-1 *Characteristics of the two batches of food waste (FW) collected and the reactor system in which they were used. HH/LO was operated at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR, LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR, and LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR. Samples were analysed in duplicates.*

Parameter	**Unit**	**FW1**	**FW2**
Reactor run		HH/LO	LH/LO, LH/HO
pH		4.05 ± 0.10	4.07 ± 0.02
Conductivity	mS cm^{-1}	6.9 ± 0.2	8.3 ± 0.4
Total Solids	% w/w	10.7 ± 0.7	11.3 ± 0.2
Volatile Solids	% w/w	8.8 ± 0.3	10.3 ± 0.2
Total COD	gCOD L^{-1}	130 ± 9	163 ± 2
Soluble COD	gCOD L^{-1}	63.7 ± 0.8	76.4 ± 1.5
Ethanol	g L^{-1}	12.7 ± 0.5	4.9 ± 0.4
Lactic acid	g L^{-1}	19.7 ± 1.3	21.7 ± 0.2
Acetic acid	g L^{-1}	4.5 ± 0.3	2.6 ± 0.2
n-Propionic acid	g L^{-1}	0.7 ± 1.0	2.1 ± 0.6

4.1.2.2. Experimental set-up and reactor operation

For each combination of OLR and HRT, a pair of 2 L glass bioreactors were operated in duplicate, each with a 0.6 L working volume, vertical mechanical stirrers (Bioprocess Control, Lund, Sweden), and regulated at 35 °C provided by a regulated warm water bath. Reactors were operated semi-continuously. A fixed volume of reactor effluent determined by the operating HRT was replaced every 3.5 days with thawed FW (Table 4-2). The FW used for LH/LO systems was diluted with tap water to obtain the required organic content

as determined by the operating OLR. After addition of fresh feed, the pH was corrected to 5.9 ± 0.1 by addition of sodium hydroxide (1 or 2 M). Gas production was monitored via water displacement using 2 L graduated glass columns containing acidified water (pH < 3.5, HCl) to avoid carbon dioxide absorption in the water, and calibrated at Standard Temperature and Pressure (STP, 273.15 K and 100 kPA). The columns were connected to the reactor headspaces via a buffer bottle to prevent liquid from the columns from entering the reactors.

Table 4-2 *Operating conditions for semi-continuous fermentation of food waste (FW) – duplicates were used.*

Condition	HRT (days)	OLR (gCOD L^{-1} d^{-1})	FW batch
HH/LO	10.4 ± 1.4	12.7 ± 1.6	1
LH/LO	8.5 ± 0.5	11.9 ± 0.6	2 (2/3 dilution)
LH/HO	8.3 ± 0.4	19.6 ± 1.0	2

At start-up, reactors were inoculated and diluted with tap water to obtain a biomass content of approx. 14 gVS L^{-1}. They were then left to acclimatise overnight to operating temperature (35 °C). During start-up for low OLR fermentations (LH/LO and HH/LO), the first feed was diluted 50% with tap water. For the higher OLR fermentation (LH/HO), the first feed cycle comprised a full feed. Day 0 of operation corresponds to the first day of feeding at full organic strength.

4.1.2.3. Evaluation of fermentation performance

To monitor fermentation performance, liquid fermentation products and pH were analysed in each effluent sample, solids and COD were measured weekly and gas composition in the water displacement columns was evaluated before feed addition (analysis see Section 4.1.2.4.). After operating for three HRT to equilibrate, cycle studies were performed to elucidate the underlying fermentation metabolism. The reactor content was sampled at structured time intervals between feeding events without opening the reactor headspace. Samples were immediately processed for product analysis. Only one of the two HH/LO reactors was subjected to a cycle study as the second reactor had ingested water from the water displacement gas measurement column in the final feeding cycle due to under pressure.

The rate of acidification was calculated as the amount of OH^- required to correct the pH in the reactor at the point of feeding, normalized over the time in between feeding events (Equation (4-1)).

$$Acidification_{(i)} = \frac{C_{NaOH(i)} \times V_{NaOH(i)}}{V_{reactor} \times (t_{(i)} - t_{(i-1)})} \text{ [mM d}^{-1}\text{]}, \quad (4\text{-}1)$$

where i represents the feeding event, C_{NaOH} is the molar concentration of the sodium hydroxide solution applied, V represents volume in L, and t represents the day of reactor operation, with $t_{(i)} - t_{(i-1)}$ being the time between feeding events. The OLR, HRT and net product yields (Y_p) were calculated for each feeding cycle as a proxy to continuous operation according Equations (4-2) and (4-3) [28].

$$OLR_{(i)} = \frac{C_{feed(i)} \times V_{feed(i)}}{V_{reactor(i)} \times (t_{(i+1)} - t_{(i)})} = \frac{C_{feed(i)}}{HRT_{(i)}} \text{ [gCOD L}^{-1}\text{ d}^{-1}\text{]}, \quad (4\text{-}2)$$

$$Y_{p(i)} = \frac{C_{p(i),effluent} - C_{p(i-1),feed}}{C_{feed}} \text{ [\%]}, \quad (4\text{-}3)$$

where C_p is the product concentration in the effluent in gCOD L^{-1} and C_{feed} is the total COD in the feed in gCOD L^{-1}. The average OLR and HRT were then obtained by averaging over the entire operation. The average product concentration, Y_p and rate of acidification were calculated by averaging the values obtained at each feeding event. This was done for each operating condition tested and started after one full HRT cycle to exclude start-up conditions. All data has been made available as a dataset [31].

4.1.2.4. Chemical analysis

C1-C4 carboxylic acids, ethanol, lactic acid, glucose were measured by high pressure liquid chromatography with a refractor index detector as previously described [26] with the oven temperature adjusted to 65 °C. The more hydrophobic MCCA (C5-C8) were analysed by gas chromatography (GC) equipped with flame ionization detector (FID), and the gas composition (N_2, O_2, H_2, CH_4 and CO_2) for the HH/LO and LH/HO fermentations were determined by GCs equipped with FID and thermal conductivity detector as previously described [28]. Due to a technical problem, composition from the biogas produced in the LH/LO system could not be analysed. Air intrusion was corrected by assuming produced biogas contains 0% O_2 and N_2.

Total solids (TS) and volatile solids (VS) were determined according to Standard Methods 2540G using fresh samples [32]. Chemical oxidation demand (COD) was measured using kits (LCK014, LCI400, Hach, Düsseldorf, Germany) before and after filtration (0.45 µm) for total and soluble COD, respectively.

4.1.2.5. Microbial community analysis

Biomass samples for microbial community analysis were taken and stored at -18°C. The inoculum was sampled in duplicate at the start-up of each duplicate reactor (i.e., n=4 for each operational condition), before the first feeding event. To evaluate the effect of

operating conditions on the microbial communities of the reactors, the final two effluent collections were sampled in each duplicate reactor and sequencing results were pooled together for bioinformatics processing (i.e., n=4 for each operational condition). Microbial samples were send to DNAsense (Aalborg Øst, Denmark) for DNA extraction, 16S rRNA gene amplicon sequencing and bioinformatics processing where reads were clustered by operational taxonomic units (OTUs), taxonomy assigned and relative abundance determined, as previously described [28]. Sequences were deposited with the ENA database (accession number PRJEB40478). The results were analysed using the DNAsense app (DNAsense, Aalborg Øst, Denmark), which is based on the *ampvis* package (v.2.5.8) in R (v. 3.5.1) [33, 34]. The number of sequencing reads per sample was between 7705 and 27323. Rarefraction curves, sequences, relative abundances and taxonomic classifications for all samples have been made available as part of a dataset [31]. The OTUs with less than 0.1% relative abundance in any sample were removed and a Non-Metric Multidimensional Scaling (NMDS) plot on the Bray-Curtis dissimilarity matrix was constructed to compare similarity of the microbial community structures in each biomass sample [35]. Alpha-diversity indices were calculated as indicator for biodiversity of the microbial communities and converted to effective diversity based on Hill numbers ^{1}D and ^{2}D [36-38].

4.1.3. Results and Discussion

4.1.3.1. Stimulating chain elongation at longer retention times

The effects of HRT and OLR were evaluated separately by comparing the performance of semi-continuous fermentation of FW in duplicate STRs. Reactors operated as HH/LO were fed with the first batch of substrate, FW1, at a HRT of 10.4 ± 1.4 days and OLR of 12.7 ± 1.6 gCOD L^{-1} d^{-1}. To determine the impact of HRT, a second batch of substrate was obtained, FW2, and diluted to allow operation of the LH/LO reactors at an HRT that was 2 days lower than HH/LO but with a similar OLR (Table 4-2). The total carboxylic acid (CA) yield was similar for HH/LO and LH/HO, i.e., Y_{CA} of 26 ± 3 % and 22 ± 4 % respectively. However, the product distribution differed (Figure 4-1).

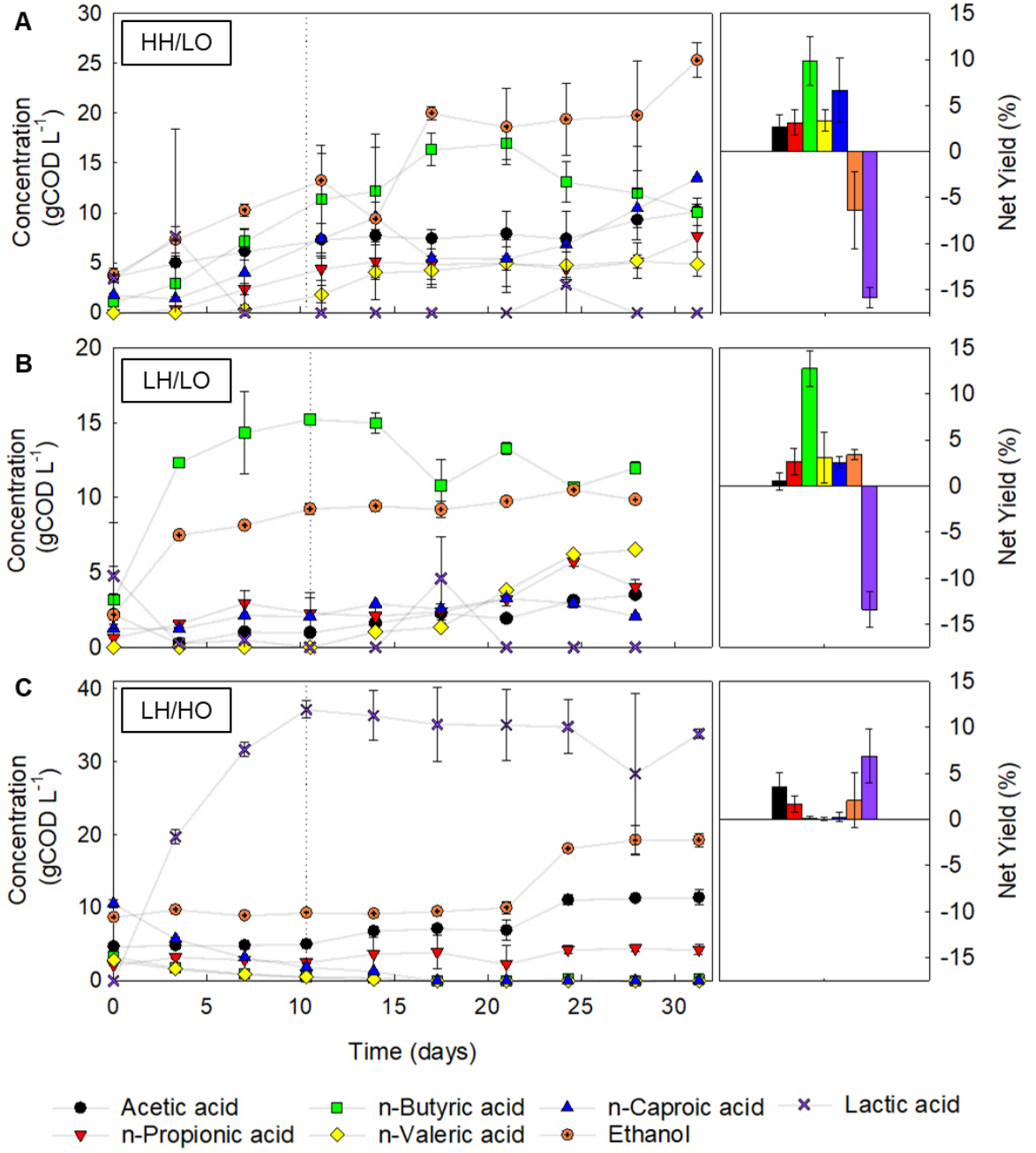


Figure 4-1 *Average concentration profiles and net yields of liquid fermentation products for three sets of operating conditions: (A) HH/LO at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR, (B) LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR, (C) LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR. Values are averaged over duplicate reactors. Net yields are determined over time from after start-up (indicated by dotted line) to end of operation.*

The LH/LO system mainly produced n-butyric acid (C4, $Y_{C4}$of 13 ± 2%), resulting in an average concentration of C4 in the effluent of 13 ± 2 gCOD L^{-1}, i.e., 55 ± 14% of the total CAs (Figure 4-1 B). In other studies focusing on butyrate-type FW fermentation selectivity was improved to 80% by, for example, control of pH at 6 [39, 40]. In the LH/LO systems in this work the pH decreased to a minimum of 5.5 between feeding events as pH was only corrected after feed addition (Figure 4-2 B). Thus, a more constant pH control might have improved selecting for C4 during fermentation.

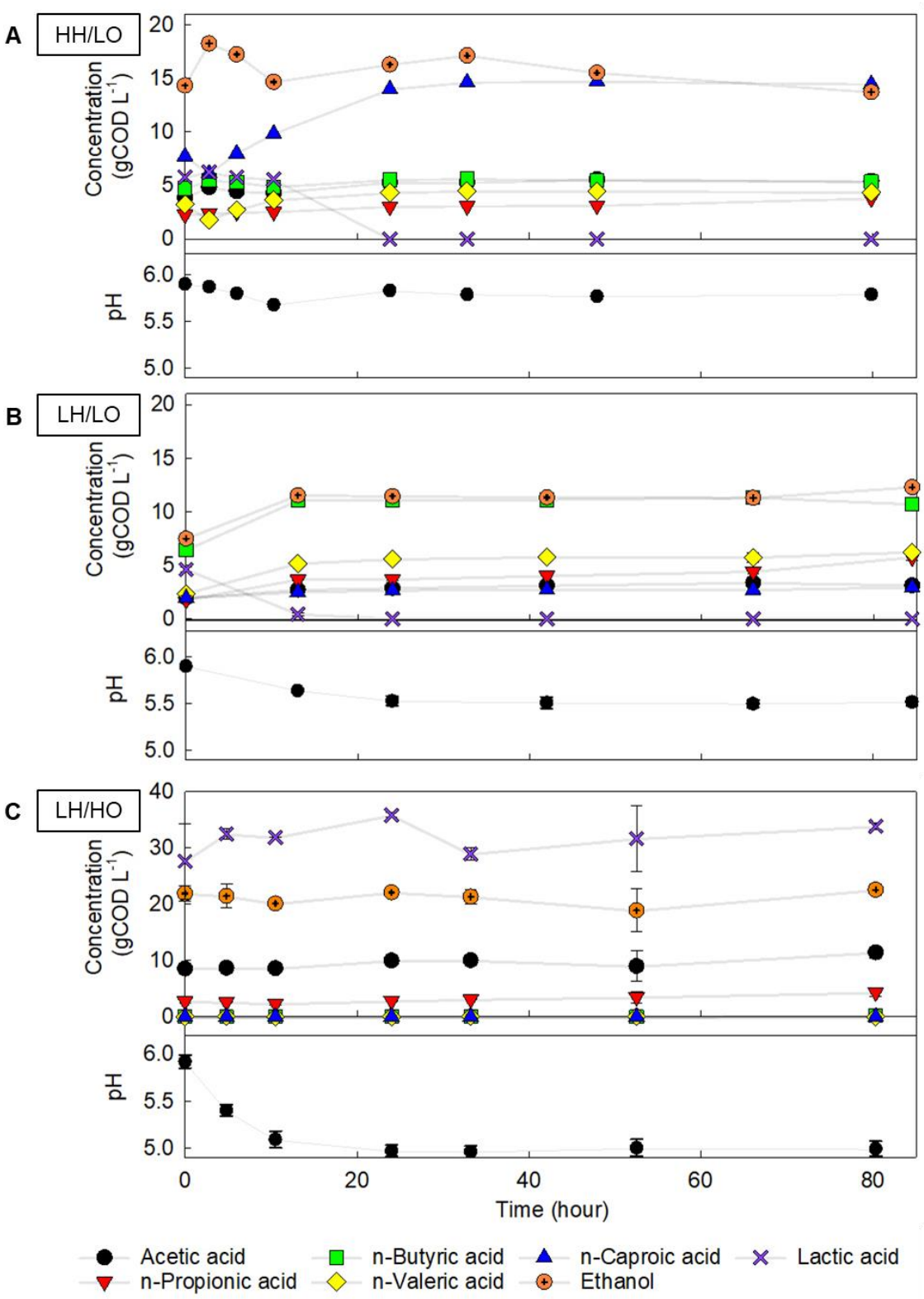


Figure 4-2 *Concentration profiles of liquid fermentation products and pH between semi-continuous feeding events. Time represents hours after the initial feed addition. Three sets of operating conditions were compared: (A) HH/LO at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR, (B) LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR, (C) LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR. Values are averaged over duplicate reactors and error bars present standard deviations (with the exception of the HH/LO system which show only 1 replicate, as the second replicate became contaminated).*

In comparison, at longer HRT (HH/LO systems) less C4 acid (Y_{C4} of 10 ± 3%) and more acetic acid (C2, Y_{C2} of 3 ± 1%) and n-caproic acid (C6, Y_{C6} of 7 ± 3%) were produced (Figure 4-1 A). Thus, a more mixed-acid metabolism was obtained in this system, where C2, C4 and C6 respectively comprised on average 21 ± 2%, 33 ± 10% and 21 ± 11% of the total CAs. The maximum C6 concentration reached in the effluent of the HH/LO reactors (13.6 gCOD L^{-1}) was over three times higher than those in the LH/LO reactors (3.4 gCOD L^{-1}). Trace concentrations of heptanoic (C7) and caprylic acid (C8) were detected near the end of operation (<0.5 gCOD L^{-1}). The higher concentrations of these MCCA in the HH/LO systems is likely from chain elongation of VFA to MCCA with ethanol and lactic acid being utilised as electron donors [41]. Ethanol accumulated under all operating conditions, as it was present in the influent (Table 4-1). However, in the HH/LO systems ethanol was net consumed ($Y_{ethanol}$ of -6 ± 4%) while in the LH/LO reactors it was net produced ($Y_{ethanol}$ of 3.4 ± 0.5%). The ethanol concentration averaged 9.7 ± 0.5 gCOD L^{-1} and 18 ± 5 gCOD L^{-1} in LH/LO and HH/LO systems, respectively, and these are concentrations which promote chain elongation [42]. For both LH/LO and HH/LO operating conditions, lactic acid from the influent (Table 4-1) was detected in the effluent at the start but was nearly fully consumed once semi-continuous operation was established.

To evaluate the fermentation mechanism, the reactor contents were sampled between feeding points after operating for 3 HRT (Figure 4-2). In the LH/LO systems lactate fell below detection limits and ethanol and CAs, mostly C4, increased in the first 13 hours after feeding (Figure 4-2 B). After, limited further fermentation was observed. In contrast, when operating at a longer HRT in the HH/LO reactors, primary acidogenic fermentation was followed by a consecutive fermentation stage of chain elongation (Figure 4-2 A). Lactic acid and VFA showed no net change for the first 10h after feed addition, however, the pH dropped to 5.68 indicating acidogenic fermentation. After 24h of fermentation, the pH recovered to 5.83 due to lactic acid being fully consumed, ethanol decreased slightly, and MCCA increased to 18.7 gCOD L^{-1}. This is in line with reports for repeated batch fermentation of FW, where initial acidogenic fermentation is followed by lactate-based chain elongation where the pH increases [13]. When lactate was depleted, no major changes were seen in the concentrations of fermentation products over the following two days. No more chain elongation took place even though ethanol and VFA were still present. Inhibition of further fermentation was not likely as the concentration of protonated C6 (maximum HC6 of 0.72 g L^{-1}), the most antimicrobial form, and ethanol remained below reported inhibitory levels (0.87 g L^{-1} HC6 and 40 g L^{-1} for ethanol) [42, 43]. It is more likely that the limited availability of lactate hindered further fermentation. Production of MCCA could probably be optimised

by operating at a higher organic load to stimulate *in situ* lactic acid production, yet without compromising on retention times, thus by using a feedstock with a higher COD content [28].

To date chain elongation in FW fermentation, without supplementation of electron donors, has predominantly been attributed to lactate as the electron donor [13]. However, several studies have reported that addition of ethanol to pre-fermented FW also resulted in chain elongation [44]. Wu et al. found that combining ethanol and lactic acid resulted in a syntrophic interaction improving MCCA production in the fermentation of Chinese liquor wastewater [62]. An initial ethanol content around 4 gCOD L^{-1} initiated chain elongation in batch fermentation of liquid FW (nearly 2 gCOD L^{-1} C6 was formed) [26]. Due to the net consumption of ethanol in the HH/LO reactors, we hypothesize that either chain elongation occurred with both the electron donors, and/or that ethanol due to its high concentration in the HH/LO systems could be oxidized to C2, resulting in a higher Y_{C2}, with co-production of H_2 [44]. The HH/LO reactors produced 0.6 ± 0.1 L $L^{-1}d^{-1}$ biogas containing 22 ± 9 % H_2. The increased level of C2 and H_2 production stimulate chain elongation, the former as a substrate and the latter to ensure a reductive environment preventing MCCA degradation [43].

4.1.3.2. Lactic acid production at elevated organic loading rate

The effect of varying OLR was evaluated by comparing the performance of the LH/LO systems to operation at higher OLR with the same HRT (the LH/HO reactors). The same substrate, FW2, was used and an increased OLR was obtained by not diluting the substrate as was done for the LH/LO systems (Table 4-2). In the LH/HO reactors, lactic acid was the dominant product (34 ± 5 gCOD L^{-1}, $Y_{lactate}$of 7 ± 2 %) (Figure 4-1 C). Ethanol also accumulated up to 14 ± 5 gCOD L^{-1}, but with $Y_{ethanol}$ near zero as it was present in the FW2 feed. Longer chain carboxylic acids, i.e., C5 and C6, were present at start-up of the LH/HO systems due to their presence in the inoculum, but they were fully washed-out after two HRT cycles. Biogas was produced at an average rate of 0.13 ± 0.09 L $L^{-1}d^{-1}$, and CO_2 comprised over 99%. Fermentation studies focusing on synthesis of lactic acid from FW reached similar outcomes, i.e., 30 to 40 gCOD L^{-1} lactic acid, but at lower HRT values of 3 to 5 days [9, 30]. Thus, yields for lactic acid might increase by shortening the HRT below 8.5 days.

The lactic acid production in the LH/HO reactors was accompanied by strong acidification. NaOH was dosed at an average of 33 ± 5 mM d^{-1} to maintain pH, whereas only 11 ± 2 and 15 ± 4 mM d^{-1} of NaOH were required for the LH/LO and HH/LO systems, respectively. In LH/HO the pH decreased rapidly in the first 12 hours after feed addition and plateaued at 5.0 (Figure 4-2 C). This was accompanied by minimal change in the concentrations of liquid

products, with only C2 and C3 increasing slightly by 2 ± 1 and 1.6 ± 0.7 gCOD L^{-1} respectively. Rapid acidification of the environment is a known outcome of the biotic activity of lactic acid bacteria (LAB), which gives them a competitive advantage over microorganisms with lower acid tolerance (Bachmann et al., 2017). Tang, et al. [45] found FW to be fermented mostly into lactic acid at pH 5, while VFA were obtained at pH 6. Because pH was not corrected during the cycle, and only at the feeding points, the acidification of the media by LAB likely allowed them to outcompete other fermentation bacteria. This effect did not take place in the LH/LO or HH/LO reactors as the pH remained above 5.5 during the entire cycle in each case (Figure 4-2).

Gu, et al. [46] noted in their leach bed reactor that a load of 100g per day of FW gave C4 as the dominant VFA product. However, when operating at 200g per day of FW the product spectrum contained mainly lactic acid. Thus, similarly, at an HRT around 8.5 days and OLR around 12 gCOD L^{-1} d^{-1} in the LH/LO system C4 was produced while at the same HRT but nearly double OLR (20 gCOD L^{-1} d^{-1}), acidogenic lactic acid-type fermentation occurred. However, the OLR was still within a suitable range for carboxylic acid fermentation (5-50 gCOD L^{-1} d^{-1}) according to literature [18]. Another study, seemingly in contrast, reported MCCA production to be stimulated in FW fermentation by an OLR around 20 gCOD L^{-1} d^{-1} because it allows to accumulate electron donors, i.e., precursors of chain elongation [28]. However, in that study HRT was almost double (14 days) than the HRT applied in the LH/HO system (8.5 days). Hence, suggesting that at insufficiently long HRT, an increase in OLR will overload the system and result in acidogenic lactic acid production. To determine if the effects observed are simply due to the operational parameter or if these have in fact selected for different microbial communities, a microbial community analysis was undertaken.

4.1.3.3. Reactor operation selected different lactic and carboxylic acid bacteria

Duplicate samples were taken from each replicate reactor before the first feeding to characterise the inoculum, and at the end of operation to characterise the microbial communities at quasi-steady state. The communities from sample duplicates, and reactor duplicates were highly similar (Figure 4-3 A). In contrast, the microbial communities from each reactor at quasi-steady state were very distinct from those of the respective inoculums, suggesting that each set of operational conditions influenced a shift in the microbiome. The cultures of the inoculum for the LH/LO reactors and of the LH/HO reactors in quasi-steady state were in proximity on the NMDS plot, which is expected since the first was inoculated from the second. However, despite the HH/LO and LH/LO reactors being given the same inoculum, i.e., effluent from an enriched chain elongation reactor fermenting FW, their

microbial cultures differed. This could be explained since before inoculation of the LH/LO system the inoculum had been stored around ten months longer at 4 °C compared to the inoculum used in HH/LO. This resulted in an alpha-diversity for the LH/LO inoculum that was less than half of that for HH/LO based on comparison of Hill numbers (Table 4-3). Interestingly, *Rummeliibacillus stabekisii*, a strictly aerobic Firmicute isolated from Antarctic soil [47], was found at high relative abundance (45 ± 3 %) in the inoculum of the LH/LO reactors. The change of microbial community composition during storage is in agreement with previous reports on preservation of microbial communities performing chain elongation [48]. Nevertheless, eventually similar communities evolved in the LH/LO and LH/HO systems and alpha-diversity increased again over operation. Thus, while storage of microbial communities for research purposes of environmental biotechnological applications remains a challenge, reactor operation eventually dictated community structure.

Table 4-3 *Biodiversity parameters of the microbial community in the inoculum and in the reactors operated according three sets of conditions (HH/LO at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR; LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR; LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR). Four biomass samples were averaged per reactor operation, i.e., two samples from each of duplicate reactors. Samples were rarefied to a depth of 10,000 reads.*

Sample	Observed OTUs	^1D	^2D	Pielou evenness
Inoculum				
HH/LO	268 ± 5	26 ± 3	8.7 ± 0.7	0.53 ± 0.02
LH/LO	151 ± 5	8.5 ± 0.6	4.0 ± 0.4	0.35 ± 0.01
LH/HO	210 ± 12	20.6 ± 0.3	10.1 ± 0.2	0.50 ± 0.00
Operation				
HH/LO	201 ± 10	24 ± 4	12 ± 3	0.52 ± 0.01
LH/LO	49 ± 5	11 ± 1	6.5 ± 0.4	0.38 ± 0.02
LH/HO	44 ± 3	8.0 ± 0.7	5.1 ± 0.9	0.34 ± 0.02

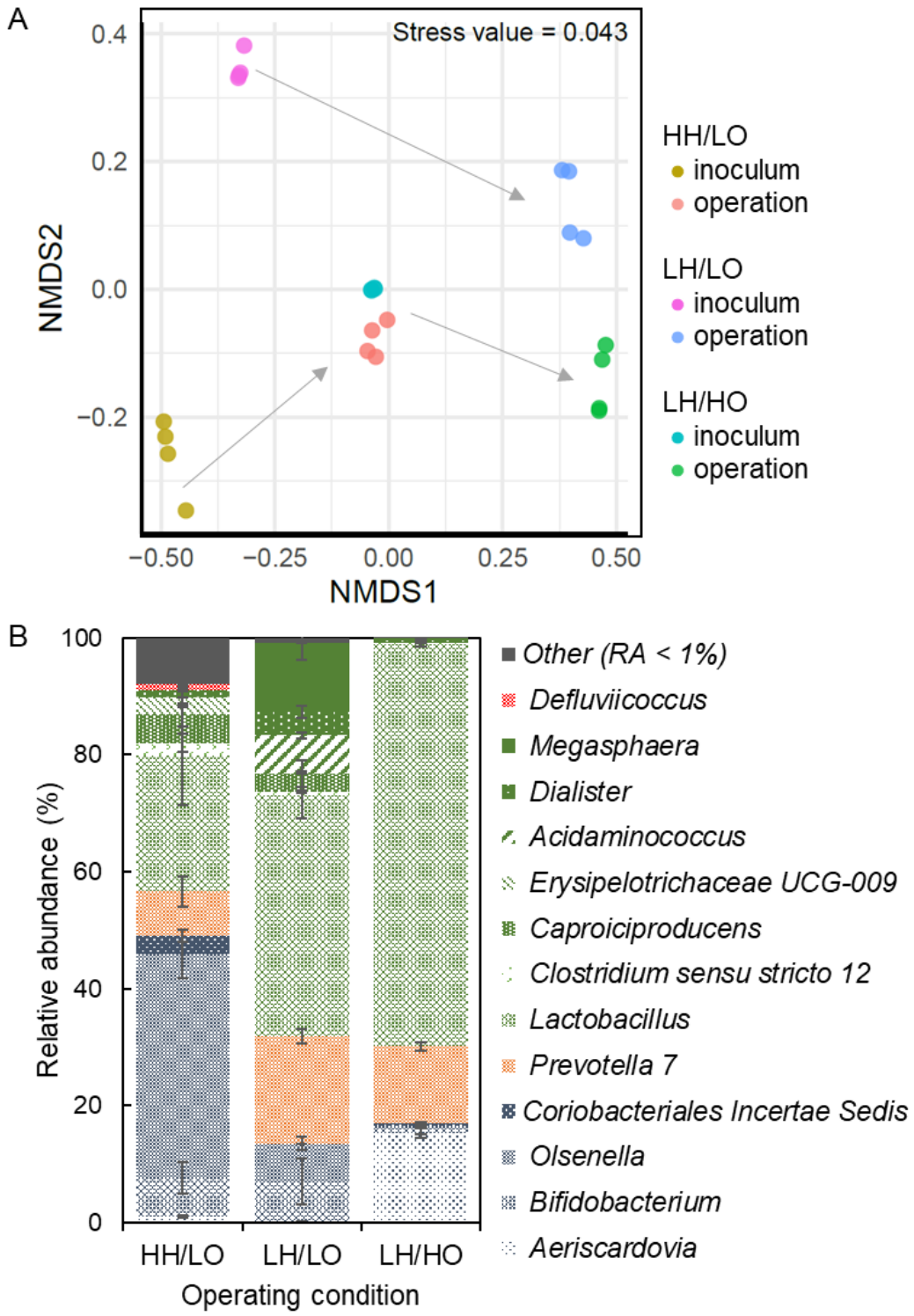


Figure 4-3 *Microbial community analysis for each set of operating conditions (n=4, 2 reactor duplicates and two sample duplicates). (A) Non-Metric Multidimensional Scaling Analysis (NMDS) based on the Bray-Curtis distance measure of 24 samples and 177 OTUs. Each point represents the community in a specific sample. The closer the sample points on the plot, the more similar the communities in the samples are. Arrows indicate the connection from inoculum to corresponding reactor culture for each set of operating condition. (B) Microbial community composition on the genus level with relative abundance >1% and coloured per phylum: red = Proteobacteria; green = Firmicutes; orange = Bacteroidetes; grey = Actinobacteria. HH/LO operation at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR, LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR, and LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR.*

Generally, less OTUs were detected in the reactor communities compared to the inocula (Table 4-3). The alpha-diversity in the reactors operated at an HRT that was two days longer (HH/LO) was double based on the first and second order Hill number ($^1D = 24 \pm 4$, $^2D = 12 \pm 3$) compared to the other systems, i.e., LH/LO and LH/HO. Thus, a more diverse community was obtained at longer HRT likely due to slower growing fermenters being able to remain in the system.

The major dominant phyla were Firmicutes, Actinobacteria and Bacteroidetes for all the three operational strategies (Figure 4-3 B). The genera with high relative abundances were mostly obligate or facultative anaerobic bacteria, tolerant to mildly acidic environments, with wide-ranging hydrolytic capabilities, and the ability to produce organic acids such as lactic acid and VFA from fermentable sugars [49-53]. Various VFA-producing genera were detected, especially in the LH/LO reactors, which is in line with their predominant functionality, i.e., C4 production. For example, *Prevotella 7* was present in all reactors but was most abundant in the LH/LO systems (Figure 4-3 B). This species converts lactic acid to VFA in FW fermentation [54]. The VFA-producing *Megasphaera* and *Acidaminococcus* were almost exclusive to the LH/LO reactors.

LAB consistently showed the highest relative abundance values. The LAB were assigned predominantly to the genus of *Lactobacillus* in the reactors operated at shorter HRT (41 ± 4 % in LH/LO and 68.8 ± 0.6 % in LH/HO). *Lactobacillus* spp. are known to be dominant in lactate-type fermentation of FW at low pH (<4.5) [54, 55].The high relative abundance of *Lactobacillus* spp. in LH/LO, but the absence of lactic acid in the effluent, indicates C4 likely resulted from lactate being metabolised [12].

In the microbial communities of the reactors, up to 31 different OTUs were classified as *Lactobacillus* spp., and these had varying relative abundances according to reactor operating conditions. The OTUs classified in the genus of *Lactobacillus* with a relative abundance > 1% were fed into a BLAST search to distinguish them according their novel genera, as recently proposed by Zheng, et al. [52]. For instance, some of the most relatively abundant *Lactobacillus* OTUs were reclassified into the novel genus of heterofermentative *Limosilactobacillus* spp, common for food fermentation where they metabolise carbohydrates into lactate, ethanol and/or C2 and CO_2 (Table 4-4).

Table 4-4 *Overview of the different OTUs assigned to the genus Lactobacillus with a minimal relative abundance of 1% in at least one of the reactors (HH/LO at 10.4 ± 1.4 days HRT and 12.7 ± 1.6 gCOD $L^{-1}d^{-1}$ OLR; LH/LO at 8.5 ± 0.5 days HRT and 11.9 ± 0.6 gCOD $L^{-1}d^{-1}$ OLR; LH/HO at 8.3 ± 0.4 days HRT and 19.6 ± 1.0 gCOD $L^{-1}d^{-1}$ OLR). The OTU sequences were run in BLAST, and the hits with highest identity match (%) were used to indicate potential reclassification of these Lactobacillus OTUs into the new classification of 25 genera proposed by Zheng, et al. [52].*

OTU_ID	Relative abundance (%)			BLAST result	Reclassification
	HH/LO	LH/LO	LH/HO		
OTU_1	11 ± 6	32 ± 2	36 ± 8	98.63% with *various L. mucosae* and *L. reuteri* strains	*Limosilactobacillus*
OTU_6	0.9 ± 0.7	3 ± 3	14 ± 7	98.63% with various *L. amylovorus* and *L. acidophilus* strains	*Lactobacillus*
OTU_27	0.1 ± 0.1	0.0 ± 0.0	6 ± 1	98.97% with unclassified homofermentative L. [56]	*Lactobacillus*
OTU_24	0.1 ± 0.2	0.0 ± 0.0	5 ± 2	98.29% with various *L. oris, L. reuteri* and *L. vaginalis* strains	*Limosilactobacillus*
OTU_171	0.5 ± 0.2	5 ± 2	1.8 ± 0.6	98.63% with various *L. mucosae* and *L. fermentum* strains	*Limosilactobacillus*
OTU_13	4 ± 2	0.0 ± 0.0	0.1 ± 0.2	97.26% with various *L. johnsonii* strains	*Lactobacillus*
OTU_9	3 ± 2	0.0 ± 0.0	0.0 ± 0.0	98.63% with various strains: *L. kimchicus*, *L. pentosiphilus*, *L. odoratitofui*, *L. silage*	*Secundilactobacillus*
OTU_35	1.7 ± 0.3	0.3 ± 0.1	0.3 ± 0.2	98.63 % with various *L. ginsenosidimutans* and *L. versmoldensis* strains	*Companilactobacillus*

The reclassification of the *Lactobacillus* genus by Zheng, et al. [52] allowed a better comparison of microbial community and function across different operating conditions (Table 4-4). For instance, in the LH/HO reactors the relative abundance of homofermentative *Lactobacillus* (e.g., OTU_6 at 14 ± 7%) was higher compared to the systems operated at lower OLR (e.g., OTU_6 at 3 ± 3% in LH/LO and <1% in HH/LO). This aligns with the high lactate yield in the LH/HO system. Another example is the almost exclusive detection of *Secundilactobacillus* spp. in the HH/LO reactors. *Secundilactobacillus* spp. generally appear in systems after primary fermenters have depleted hexoses and disaccharides, where they perform secondary fermentation of metabolizing pentoses to pyruvate. Thus, by operating at an HRT of two days longer, secondary fermentation was stimulated which was reflected in the microbial community.

Caproiciproducens spp. was detected in the HH/LO (5 ± 2%) and LH/LO (3 ± 2%) systems but not in the LH/HO reactor. The sequence of the OTU assigned to the genus *Caproiciproducens* with the highest relative abundance was closely similar to the bacteria *Ruminococcaceae* CPB6, found in several lactic acid-based chain elongation systems (98.63% identity match in BLAST) [57, 58]. *Caproiciproducens* spp. belongs to a distinct lineage of *Clostridium* group IV in the family of Ruminococcaceae, and can produce ethanol, lactic acid, C2, C4 and C6 via chain elongation [59, 60]. It was enriched during cheese whey fermentation for VFA production by increasing SRT from 10 to 15 days [61]. Thus, operating at longer HRT in the HH/LO selected for a higher relative abundance of *Caproiciproducens* spp. However, this genus was also detected at lower HRT in LH/LO, thus at the lower HRT it did not fully wash out. Its absence (undetected) in the LH/HO system suggests that at higher OLR it was unable to compete in the microbial community of acidogenic lactate-fermentation.

Caproiciproducens was not the only genus found that relates to C6 production. Some OTUs were assigned to *Clostridium sensu stricto 12* with a relative abundance larger than 1% only found in the HH/LO systems. Members of this genus have been found to either produce C6 via ethanol-based chain elongation or compete with it by excessive production of C4 [48, 58, 62]. The presence of both genera that contain chain-elongating species using both lactic acid and ethanol strengthens the hypothesis that both types of chain elongation could take place.

Other prominent LAB included *Olsenella*, which had the highest relative abundance (38 ± 4%) outscoring *Lactobacillus* spp. (22 ± 8 %) in the HH/LO reactors. *Olsenella* spp. were also detected in the LH/LO systems but to a lesser extent (6 ± 1 %), whilst in the LH/HO reactors the relative abundance was below 1%. This genus ferments carbohydrates to

predominantly lactic acid and is linked with hydrolysis and primary fermentation in chain elongating systems, usually co-occurring with the chain-elongating *Pseudoramibacter* spp. [50, 58, 63]. However, the latter were not detected here.

The other LAB detected were part of the family of Bifidobacteriaceae where the dominant genus depended on which OLR was employed. *Bifidobacterium* spp. had a relative abundance of 7 ± 4% in the LH/LO and HH/LO systems but were less than 1% when operating at higher OLR (LH/HO reactors). In contrast, *Aeriscardovia* spp. were the second most abundant in the LH/HO reactors (15.3 ± 0.9%) but showed < 1.1 % in the reactors operated at lower OLR, i.e., LH/LO and HH/LO. They are oxygen and acid tolerant bacteria that produce C2 and lactate, and were found before in lactic acid fermentation from FW and lactate-based chain elongation reactors [30, 58, 63]. Thus, operating at elevated OLR or HRT selected for different types of LAB, each linked to different fermentation pathways to lactic acid, C4 or C6 and in accordance with the kinetic profiles and phenotypes observed for each set of reactors.

4.1.4. Conclusion

This study has demonstrated how separately manipulating only two of the key operational parameters in semi-continuous STR, i.e., the HRT and OLR, is sufficient to stimulate three different pathways in FW fermentation. n-Butyric acid was the main product at the lowest HRT and OLR tested (8.5 d and 12 gCOD $L^{-1}d^{-1}$). An HRT of two days longer stimulated secondary fermentation mostly to n-caproic acid from chain elongation. Instead, operating at higher OLR (around 20 gCOD $L^{-1}d^{-1}$) while using a similar HRT (8.5 d) led to lactate-type fermentation. This metabolic shift was reflected in the microbial communities. Longer HRT allowed for higher relative abundances of Olsenella spp., *Secundilactobacillus* spp. and species related to chain elongation. Operating at higher OLR resulted, instead, in higher relative abundances of homofermentative *Lactobacillus* spp.. Thus, to target n-butyric acid in FW fermentation: operating at lower OLR can avert excessive lactic acid production; and a lower HRT can limit secondary fermentation. Targeting lactic acid requires a higher OLR whereby rapid acidification allows homofermentative *Lactobacillus* spp. to outcompete other fermentative bacteria. When the goal is MCCA production, a sufficient HRT is required for consecutive secondary fermentation such as chain elongation. These findings provide an essential contribution on how OLR and HRT can serve as key parameters in the design of a flexible acidogenic FW fermentation platform. The possibility of having an adaptable product range according to market demand by repurposing existing single stage AD assets will allow integrating this technology within waste management systems, and hence accelerate a broader range of circular economy outcomes from FW valorisation.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement [H2020-MSCA-CO-FUND 665992]; by the EPSRC [EP/L016354/1]; and Wessex Water and GENeco (Bristol, UK).

Acknowledgements

The authors would like to thank Tom Phelps and Wesley Wong from GENeco for reliable and enthusiastic support of the research performed in the supply of samples and information. We would also like to acknowledge the DNAsense staff for their insightful guidance and assistance regarding the 16s rRNA gene amplicon sequencing. We would also like to extend our thanks to our colleagues at the Environmental Engineering Cluster at the Singapore Centre for Life Science and Engineering for some helpful discussions regarding microbial community analysis.

References

[1] European Commission, "COM/2019/190 final, on the implementation of the circular economy action plan," Brussels, 2019.

[2] D. Gasper, A. Shah, and S. Tankha, "The Framing of Sustainable Consumption and Production in SDG 12," *Global Policy,* vol. 10, no. S1, pp. 83-95, 2019.

[3] K. M. Kibler, D. Reinhart, C. Hawkins, A. M. Motlagh, and J. Wright, "Food waste and the food-energy-water nexus: A review of food waste management alternatives," *Waste Manag,* vol. 74, pp. 52-62, Apr 2018.

[4] J. Edwards, M. Othman, E. Crossin, and S. Burn, "Life cycle inventory and mass-balance of municipal food waste management systems: Decision support methods beyond the waste hierarchy," *Waste Manag,* vol. 69, pp. 577-591, Nov 2017.

[5] A. S. Engelberth, "Evaluating economic potential of food waste valorization: Onward to a diverse feedstock biorefinery," *Current Opinion in Green and Sustainable Chemistry,* vol. 26, 2020.

[6] S. Venkata Mohan *et al.*, "Waste biorefinery models towards sustainable circular bioeconomy: Critical review and future perspectives," *Bioresour Technol,* vol. 215, pp. 2-12, Sep 2016.

[7] R. Moscoviz, E. Trably, N. Bernet, and H. Carrère, "The environmental biorefinery: state-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation," *Green Chemistry,* vol. 20, no. 14, pp. 3159-3179, 2018.

[8] G. Capson-Tojo, M. Rouez, M. Crest, J.-P. Steyer, J.-P. Delgenès, and R. Escudié, "Food waste valorization via anaerobic processes: a review," *Reviews in Environmental Science and Bio/Technology,* vol. 15, no. 3, pp. 499-547, 2016.

[9] J. Tang, X. Wang, Y. Hu, Y. Zhang, and Y. Li, "Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR," *Waste Manag,* vol. 52, pp. 278-85, Jun 2016.

[10] L. Zhang, K. C. Loh, Y. Dai, and Y. W. Tong, "Acidogenic fermentation of food waste for production of volatile fatty acids: Bacterial community analysis and semi-continuous operation," *Waste Manag,* vol. 109, pp. 75-84, May 15 2020.

[11] S. Im, M.-K. Lee, Y.-M. Yun, S.-K. Cho, and D.-H. Kim, "Effect of storage time and temperature on hydrogen fermentation of food waste," *International Journal of Hydrogen Energy,* vol. 45, no. 6, pp. 3769-3775, 2020.

[12] F. Asunis *et al.*, "Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey," *Bioresour Technol,* vol. 289, p. 121722, Oct 2019.
[13] C. A. Contreras-Davila, V. J. Carrion, V. R. Vonk, C. N. J. Buisman, and D. Strik, "Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation," *Water Res,* vol. 169, p. 115215, Feb 1 2020.
[14] Y. S. Jang *et al.*, "Bio-based production of C2-C6 platform chemicals," *Biotechnol Bioeng,* vol. 109, no. 10, pp. 2437-59, Oct 2012.
[15] J. Kannengiesser, K. Sakaguchi-Soder, T. Mrukwia, J. Jager, and L. Schebek, "Extraction of medium chain fatty acids from organic municipal waste and subsequent production of bio-based fuels," *Waste Manag,* vol. 47, no. Pt A, pp. 78-83, Jan 2016.
[16] S. K. Bhatia and Y. H. Yang, "Microbial production of volatile fatty acids: current status and future perspectives," *Reviews in Environmental Science and Bio/Technology,* vol. 16, no. 2, pp. 327-345, 2017.
[17] F. Girotto, M. C. Lavagnolo, A. Pivato, and R. Cossu, "Acidogenic fermentation of the organic fraction of municipal solid waste and cheese whey for bio-plastic precursors recovery - Effects of process conditions during batch tests," *Waste Manag,* vol. 70, pp. 71-80, Dec 2017.
[18] D. Arslan *et al.*, "Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations," *Critical Reviews in Environmental Science and Technology,* vol. 46, no. 6, pp. 592-634, 2016.
[19] M. Zhou, B. Yan, J. W. C. Wong, and Y. Zhang, "Enhanced volatile fatty acids production from anaerobic fermentation of food waste: A mini-review focusing on acidogenic metabolic pathways," *Bioresour Technol,* vol. 248, no. Pt A, pp. 68-78, Jan 2018.
[20] D. Nguyen, V. Gadhamshetty, S. Nitayavardhana, and S. K. Khanal, "Automatic process control in anaerobic digestion technology: A critical review," *Bioresour Technol,* vol. 193, pp. 513-22, Oct 2015.
[21] A. Bensmann, R. Hanke-Rauschenbach, and K. Sundmacher, "Reactor configurations for biogas plants – a model based analysis," *Chemical Engineering Science,* vol. 104, pp. 413-426, 2013.
[22] H. Kim, J. Kim, S. G. Shin, S. Hwang, and C. Lee, "Continuous fermentation of food waste leachate for the production of volatile fatty acids and potential as a denitrification carbon source," *Bioresour Technol,* vol. 207, pp. 440-5, May 2016.
[23] I. Vanwonterghem, P. D. Jensen, K. Rabaey, and G. W. Tyson, "Temperature and solids retention time control microbial population dynamics and volatile fatty acid production in replicated anaerobic digesters," *Sci Rep,* vol. 5, p. 8496, Feb 16 2015.
[24] I. O. Bolaji and D. Dionisi, "Acidogenic fermentation of vegetable and salad waste for chemicals production: Effect of pH buffer and retention time," *Journal of Environmental Chemical Engineering,* vol. 5, no. 6, pp. 5933-5943, 2017.
[25] E. Jankowska, J. Chwialkowska, M. Stodolny, and P. Oleskowicz-Popiel, "Effect of pH and retention time on volatile fatty acids production during mixed culture fermentation," *Bioresour Technol,* vol. 190, pp. 274-80, Aug 2015.
[26] M. Coma *et al.*, "Organic waste as a sustainable feedstock for platform chemicals," *Faraday Discuss,* vol. 202, pp. 175-195, Sep 21 2017.
[27] M. A. Khan *et al.*, "Optimization of process parameters for production of volatile fatty acid, biohydrogen and methane from anaerobic digestion," *Bioresour Technol,* vol. 219, pp. 738-748, Nov 2016.
[28] V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting Organic Load as a Strategy to Direct Single-Stage Food Waste Fermentation from Anaerobic Digestion to Chain Elongation," *Processes,* vol. 8, no. 11, 2020.
[29] Z. Bo and H. Pin-Jing, "Performance assessment of two-stage anaerobic digestion of kitchen wastes," *Environ Technol,* vol. 35, no. 9-12, pp. 1277-85, May-Jun 2014.

[30] Y. Wu, H. Ma, M. Zheng, and K. Wang, "Lactic acid production from acidogenic fermentation of fruit and vegetable wastes," *Bioresour Technol,* vol. 191, pp. 53-8, Sep 2015.
[31] V. De Groof, M. Coma Bech, T. Arnot, D. Leak, and A. lanham, "Dataset for "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters"," ed. Bath: University of Bath Research Data Archive, 2020.
[32] APHA, "Standard methods for the examination of water and wastewater," vol. 23, 2017.
[33] R Core Team, "R: A language and envionment for statistical computing," ed. Vienna, Austria: R Foundation for Statistical Computing, 2017.
[34] M. Albertsen, S. M. Karst, A. S. Ziegler, R. H. Kirkegaard, and P. H. Nielsen, "Back to Basics-The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities," *PLoS One,* vol. 10, no. 7, p. e0132783, 2015.
[35] R. J. Bray and J. T. Curtis, "An ordination of the upland forest community of southern Wisconsin," *Ecological Monographs,* vol. 27, no. 4, pp. 325-349, 1957.
[36] R. Lucas, J. Groeneveld, H. Harms, K. Johst, K. Frank, and S. Kleinsteuber, "A critical evaluation of ecological indices for the comparative analysis of microbial communities based on molecular datasets," *FEMS Microbiol Ecol,* vol. 93, no. 1, Jan 2017.
[37] S. H. Hurlbert, "The nonconcept of species diversity: a critique and alternative parameters," *Ecology,* vol. 52, pp. 577-586, 1971.
[38] L. Jost, "Entropy and diversity," *Oikos,* vol. 113, no. 2, pp. 363-375, 2006.
[39] K. Wang, J. Yin, D. Shen, and N. Li, "Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: effect of pH," *Bioresour Technol,* vol. 161, pp. 395-401, Jun 2014.
[40] Y. Jin *et al.*, "Volatile fatty acids production from saccharification residue from food waste ethanol fermentation: Effect of pH and microbial community," *Bioresour Technol,* vol. 292, p. 121957, Nov 2019.
[41] C. M. Spirito, H. Richter, K. Rabaey, A. J. Stams, and L. T. Angenent, "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr Opin Biotechnol,* vol. 27, pp. 115-22, Jun 2014.
[42] S. Lonkar, Z. Fu, and M. Holtzapple, "Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate," *Biotechnol Bioeng,* vol. 113, no. 12, pp. 2597-2604, Dec 2016.
[43] L. T. Angenent *et al.*, "Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals," *Environ Sci Technol,* vol. 50, no. 6, pp. 2796-810, Mar 15 2016.
[44] M. Roghair *et al.*, "Controlling Ethanol Use in Chain Elongation by CO2 Loading Rate," *Environ Sci Technol,* vol. 52, no. 3, pp. 1496-1505, Feb 6 2018.
[45] J. Tang, X. C. Wang, Y. Hu, Y. Zhang, and Y. Li, "Effect of pH on lactic acid production from acidogenic fermentation of food waste with different types of inocula," *Bioresour Technol,* vol. 224, pp. 544-552, Jan 2017.
[46] X. Y. Gu, J. Z. Liu, and J. W. C. Wong, "Control of lactic acid production during hydrolysis and acidogenesis of food waste," *Bioresour Technol,* vol. 247, pp. 711-715, Jan 2018.
[47] F. Faira da Mota, R. E. Vollu, D. Jurelevicius, and L. Seldin, "Whole-Genome Sequence of Rummeliibacillus stabekisii Strain PP9 Isolated from Antarctic Soil," *Genome Announc,* vol. 4, no. 3, May 26 2016.
[48] P. Candry, S. Huang, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Enrichment and characterisation of ethanol chain elongating communities from natural and engineered environments," *Sci Rep,* vol. 10, no. 1, p. 3682, Feb 28 2020.

[49] N. R. Krieg, W. Ludwig, J. Euzéby, and W. B. Whitman, "Phylum XIV. Bacteroidetes phyl. nov.," in *Bergey's Manual® of Systematic Bacteriology*, N. R. Krieg *et al.*, Eds. New York: Springer, 2010.
[50] M. Kraatz, R. J. Wallace, and L. Svensson, "Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of Olsenella, Olsenella uli and Olsenella profusa," *Int J Syst Evol Microbiol,* vol. 61, no. Pt 4, pp. 795-803, Apr 2011.
[51] P. Mattarelli and B. Biavati, "Genus II. Aeriscardovia Simpson, Ross, Fitzgerald and Stanton 2004, 405," in *Bergey's manual® of Systematic Biology: The Actinobacteria, Part A and B*, vol. 5, M. Goodfellow *et al.*, Eds. 2 ed.: Springer, 2012, pp. 206-207.
[52] J. Zheng *et al.*, "A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae," *Int J Syst Evol Microbiol,* Apr 15 2020.
[53] A. C. Parte *et al.*, *Bergey's manual® of Systematic Bacteriology: The Firmicutes*, 2 ed. Springer, 2009.
[54] K. Feng, H. Li, and C. Zheng, "Shifting product spectrum by pH adjustment during long-term continuous anaerobic fermentation of food waste," *Bioresour Technol,* vol. 270, pp. 180-188, Dec 2018.
[55] F. Bonk, J. R. Bastidas-Oyanedel, A. F. Yousef, and J. E. Schmidt, "Exploring the selective lactic acid production from food waste in uncontrolled pH mixed culture fermentations using different reactor configurations," *Bioresour Technol,* vol. 238, pp. 416-424, Aug 2017.
[56] B. K. Acharya, H. Pathak, S. Mohana, Y. Shouche, V. Singh, and D. Madamwar, "Kinetic modelling and microbial community assessment of anaerobic biphasic fixed film bioreactor treating distillery spent wash," *Water Res,* vol. 45, no. 14, pp. 4248-59, Aug 2011.
[57] X. Zhu *et al.*, "Production of high-concentration n-caproic acid from lactate through fermentation using a newly isolated Ruminococcaceae bacterium CPB6," *Biotechnol Biofuels,* vol. 10, p. 102, 2017.
[58] B. Liu, S. Kleinsteuber, F. Centler, H. Harms, and H. Strauber, "Competition Between Butyrate Fermenters and Chain-Elongating Bacteria Limits the Efficiency of Medium-Chain Carboxylate Production," *Front Microbiol,* vol. 11, p. 336, 2020.
[59] B. C. Kim, B. Seung Jeon, S. Kim, H. Kim, Y. Um, and B. I. Sang, "Caproiciproducens galactitolivorans gen. nov., sp. nov., a bacterium capable of producing caproic acid from galactitol, isolated from a wastewater treatment plant," *Int J Syst Evol Microbiol,* vol. 65, no. 12, pp. 4902-4908, Dec 2015.
[60] M. Flaiz, T. Baur, S. Brahner, A. Poehlein, R. Daniel, and F. R. Bengelsdorf, "Caproicibacter fermentans gen. nov., sp. nov., a new caproate-producing bacterium and emended description of the genus Caproiciproducens," *Int J Syst Evol Microbiol,* Jun 25 2020.
[61] B. Lagoa-Costa, C. Kennes, and M. C. Veiga, "Cheese whey fermentation into volatile fatty acids in an anaerobic sequencing batch reactor," *Bioresour Technol,* vol. 308, p. 123226, Jul 2020.
[62] Q. Wu *et al.*, "Upgrading liquor-making wastewater into medium chain fatty acid: Insights into co-electron donors, key microflora, and energy harvest," *Water Res,* vol. 145, pp. 650-659, Nov 15 2018.
[63] J. Lambrecht *et al.*, "Key sub-community dynamics of medium-chain carboxylate production," *Microb Cell Fact,* vol. 18, no. 1, p. 92, May 28 2019.

Chapter 5. Feeding pattern affects balance between lactic acid fermentation and microbial chain elongation

In the discussion of the literature review, it was hypothesized that chain elongation might be stimulated in acidogenic food waste fermentation by operating with a semi-continuous feeding pattern instead of continuous feeding. The idea was further explored in this chapter by comparing feeding patterns. This is in line with the third research objective of this thesis: assessing the effect of a semi-continuous feeding pattern on production of MCCA. Furthermore, to understand how feeding pattern affected fermentation, the microbial community and fermentation pathways were analysed, in line with research objectives five and six respectively.

To allow for more continuous feeding and relate the immediate impacts to the mixed culture a more advanced reactor system and knowledge regarding the analysis of MMC was required. Therefore, a collaboration was set up with the Environmental Engineering cluster under the supervision of Prof. Stefan Wuertz at the Singapore Centre for Environmental Life Sciences Engineering (SCELSE, NTU, Singapore). The work presented in this chapter was performed as part of a four-month research placement.

Some additional technical assistance was received for reactor operation, sample analysis and microbial community analysis. Abeed Fatima Binti Mohidin Batcha from SCELSE performed DNA extraction, library preparation, sending for sequencing and initial bioinformatics processing up to the generation of an amplicon sequence variant table. The microbial community statistics presented in the current work have been performed by the author of the thesis with guidance and examples received from experts at SCELSE.

This chapter is submitted in an alternative thesis format in line with Appendix 6A of the “Specifications for Higher Degree Theses and Portfolios” as required by the University of Bath.

5.1. Prepared manuscript: Feeding pattern affects balance between lactic acid fermentation and microbial chain elongation

Vicky De Groof[1,2], Abeed Fatima Binti Mohidin Batcha[3], Ezequiel Santillan[3], Marta Coma[4], Tom Arnot[2,4,5], David J Leak[4,5,6], Stefan Wuertz[3], Ana B Lanham[2,4,5]*

[1] EPSRC Centre for Doctoral Training in Sustainable Chemical Technologies, University of Bath, Claverton Down, Bath BA2 7AY, UK;
[2] Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK;
[3] Singapore Centre for Environmental Life Sciences Engineering (SCELSE), School of Civil and Environmental Engineering, Nanyang Technological University, Singapore;
[4] Centre for Sustainable Chemical Technologies (CSCT), University of Bath, Claverton Down, Bath BA2 7AY, UK;
[5] Water Innovation & Research Centre (WIRC), University of Bath, Claverton Down, Bath BA2 7AY, UK;
[6] Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK;
* Correspondence: A.Lanham@bath.ac.uk ; Tel.: +441225384544

Abstract

Acidogenic mixed culture fermentation for the production of medium chain carboxylic acids (MCCA) via microbial chain elongation is a promising technology to improve bio-waste recycling. However, the lack in understanding on how to control the various possible fermentation pathways is limiting product selectivity and yields. Here, we show that feeding pattern affected the microbial community composition and fermentation outcome in single-stage food waste fermentation. Co-fermentation of food waste with soybean soaking wastewater resulted in the predominant production of lactic acid, ethanol, and even-chained MCCA where n-caproic and n-caprylic acid were produced at average concentrations of 16 ± 6 gCOD L^{-1} and 8 ± 3 gCOD L^{-1}, respectively. Longer batch cycles, as evaluated by a bi-weekly feeding pattern, resulted in a more stable product profile where a balance of lactic acid, ethanol and acetate fermentation was followed by chain elongation. In comparison, shorter batch cycles, i.e., daily feeding, attained similar yields of overall liquid fermentation products, yet the process was more unstable due to more frequent peaks of lactic acid concentration in the effluent which went up to 43 gCOD L^{-1}. In addition, nearly double the amount of pH-correcting chemicals were required when operating at a daily feeding pattern. Thus, longer cycles in semi-continuous stirred tank reactors (sCSTR) operation improved not only process stability but also reduced the need for chemical addition. Statistical tests on reactor performance and evaluation of the microbial community through 16S rRNA gene amplicon sequencing revealed two competing fermentation pathways. Namely, a syntrophic

fermentation pathway whereby primary fermentation with production of lactic acid, ethanol and volatile fatty acids was followed by chain elongation on one side, and homolactic fermentation on the other. Daily reactor feeding provided homolactic *Lactobacillus* spp. with a competitive advantage over other fermentative bacteria by the more frequent introduction of highly biodegradable content. These results highlight how understanding competitive and syntrophic interactions in fermentative microbial communities can be used to improve process design for MCCA production.

5.1.1. Introduction

Recovery of energy and bio-chemicals from unavoidable bio-waste, creates opportunities to use resources in a more economic, effective and environmentally friendly way [1]. Food waste (FW) is a bio-waste with a high organic and water content and its disposal and treatment pose a health and environmental risk. For example, traditional FW management, such as landfilling and incineration, results in the emissions of greenhouse gasses and odours via natural fermentation [2, 3].

Controlled anaerobic fermentation with microbial communities is an attractive bio-waste valorisation technology. Because distinct metabolic pathways co-exist, different fermentation compounds can be recovered that have application as energy carriers or as chemical building blocks, for instance, biogas from anaerobic digestion (AD) [4]. In microbial culture fermentation of FW, hydrolysis products, i.e., organic monomers, are converted to a mixture of ethanol, volatile fatty acids (VFA, carboxylic acids with 1 to 5 carbon atoms), hydrogen and/or lactic acid [5, 6]. Lactate-type fermentation can be further categorized into homolactic or heterolactic fermentation where, respectively, either only lactic acid or a combination of acetic acid and/or ethanol with lactic acid is obtained [7]. Consecutive fermentation steps can occur, for instance, lactic acid can be subsequently degraded to VFA and H_2 [8], and/or VFA can undergo chain elongation with lactic acid and/or ethanol as electron donor to medium chain carboxylic acids (MCCA) [9]. MCCA are of particular interest due to their hydrophobic properties, potentially easing separation, and higher energetic and monetary value compared to other fermentation intermediates.

Production of MCCA via microbial chain elongation requires a sufficient cell retention time in the bioreactor, inhibition of methanogenesis and reductive conditions [10]. Systems with biomass retention typically used for MCCA production are unsuitable for FW. This is due to FW generally having a high solids content (228 ± 100 g $kg_{wet\ weight}^{-1}$ of total solids, TS [11]) leading to problems with pumping or solids accumulation. Therefore, previous lab-scale research aiming at MCCA production from FW in a single-stage system used semi-continuous fermentation in leach bed or sequential batch stirred tank reactors [12, 13].

During such batch-like, semi-continuous operation, the accumulation of electron donors during primary fermentation is followed by chain elongation, resulting over time in a MCCA-producing microbial community [14].

Limited information is available on the impact of operating with semi-continuous feeding on fermentation with anaerobic microbial communities. Even so, such feeding strategies will affect the way resources are available to the microbial community in the reactors. Differences in resource availability will shape the various competitive and cooperative interactions in microbial communities [15]. In other biotechnological processes relying on microbial communities, such as production of polyhydroxyalkanoates, dynamic feeding patterns influence process performance [16]. Some studies mention the impact of the duration of a sequential batch for lactic acid or VFA production [8, 17]. Studies on the feeding pattern for AD note that frequent short feeds or even continuous operation of a diluted feed is optimal as it creates a nearly imperceptible organic overload [18]. Decreasing the frequency of feeding events in AD, but maintaining the overall average organic loading rate (OLR), leads to larger fluctuations in actual organic load as higher substrate loads are introduced per feed event, and this causes VFA accumulation and methanogenesis inhibition [19, 20]. Similarly, we hypothesise that by operating with a semi-continuous feeding pattern to concentrate the organic load at fewer feeding events, the precursors of chain elongation, i.e., VFA, lactic acid and ethanol can more readily accumulate and provide the reductive conditions to promote chain elongation. Thus, the aim of the current work was to explore the impact of feeding pattern on chain elongation in FW fermentation. Four anaerobic bioreactors co-fermenting a mixture of FW and soybean processing wastewater were set up to compare the impact of feeding daily versus feeding twice a week on fermentation performance and microbial community composition. The aim of this evaluation was to shed light on optimal feeding strategies to maximise production of value-added compounds from FW via chain elongation.

5.1.2. Materials and methods

5.1.2.1. Feedstock and inoculum

Fresh solid FW was collected twice a week from a local cafeteria (Nanyang Technological University, Singapore). The FW was mixed with food processing wastewater to obtain a pumpable feedstock. Specifically, we used soybean soaking wastewater (Soy) obtained after soaking yellow soybeans for four hours, which was collected weekly from a soybean processing plant and stored at 4 °C (Mr. Bean, Singapore). Upon collection of the FW, hard residuals such as animal bones or shells were manually removed. Then the fresh FW was shredded and mixed with Soy in an electrical blender. Two mass ratios of FW/Soy were

used, namely 0.5 kg L^{-1} and 0.38 kg L^{-1}, to obtain tCOD contents of 132 ± 19 gCOD L^{-1} and 90 ± 19 gCOD L^{-1}, respectively. Feedstock characterisation, variability and degradation during storage were evaluated (available in supplementary information Table S5-1 and Table S5-2). Six samples of fresh feedstock were taken and stored (- 80 °C) for subsequent characterisation of the community in the feed.

Reactors were inoculated with fresh AD effluent from a wastewater treatment plant (pH = 7.19 ± 0.02, VS = 21.97 ± 0.05 g L^{-1}) (Changi, Singapore). Inoculum was diluted with tap water to obtain 10 gVS L^{-1} in each reactor and acclimated at an operating temperature of 35 °C for 3 days to remove residual organics from the seeding sludge. Before the first feeding event, a sample was taken of the acclimated inoculum in each reactor and stored (- 80 °C) to determine its microbial composition.

5.1.2.2. Bioreactor setup and operation

Four 5 L double-jacketed semi-continuous stirred tank reactors (sCSTR) were operated in duplicate pairs (3 L working volume, 35 °C). Two reactors were fed bi-weekly ($sCSTR_{BW}$ 1 and 2) and two reactors were fed daily ($sCSTR_{D}$ 1 and 2). Other operational parameters were kept the same. The reactors operated at an average hydraulic retention time (HRT) of 10.5 days. The pH was controlled at a minimum of 5.5 by automated dosing of 2 M NaOH to prevent acid inhibition. After a period equating to 8 HRT, the $sCSTR_{D}$ reactors were switched to bi-weekly feeding following $sCSTR_{BW}$ operation ($sCSTR_{BW\text{-}new}$ 1 and 2).

Start-up occurred by feeding all reactors with an organic overload, with a feed-to-microorganism ratio of 5 gCOD gVS^{-1}, and then letting fermentation take place over 3.5 days to inhibit methanogenesis and initiate acidogenic fermentation. After the first feeding event, sCSTR operation began at Day 0 of operation. During reactor feeding a fixed volume of reactor content was replaced with a fixed volume of feedstock either daily or twice a week. Feedstock was prepared within 24 hours of feeding $sCSTR_{BW}$ and kept at 4 °C for up to 3 days for feeding $sCSTR_{D}$. The FW/Soy ratio in the feedstock was 0.5 kg L^{-1} during the first 70 days of operation, leading to an organic loading rate (OLR) of 12.6 ± 2.0 gCOD $L^{-1}d^{-1}$. From Day 70 of operation, the FW/Soy ratio was changed to 0.38 kg L^{-1} to reduce the organic load (OLR of 8.6 ± 1.8 gCOD $L^{-1}d^{-1}$) since it was noticed that lactic acid instead of chain elongation products started accumulating in the effluent. Our previous study had reported that operating at lower OLR selects for carboxylic acids over lactic acid in acidogenic FW fermentation [21]. Average operating conditions per reactor are outlined in Table S5-3. The start-up phase was defined as the period required to reach a stable volatile solids (VS) content in the reactor effluent (< 20% variation), which was found to be after two HRTs (Figure S5-1).

The OLR, HRT and product yields (Y_p) were determined for each feeding event as an average over the fermentation cycle, to allow comparison between the different feeding patterns. The HRT was calculated from the influent flow rate (Q), determined from the feeding volume (V_{feed}) and duration of fermentation cycle (Equation (5-1)). The OLR was calculated using the total COD (tCOD) content measured for each prepared feedstock, i.e., twice a week (C_{feed})(Equation (5-2)). Reactor performance was evaluated by calculating the yield of liquid fermentation products, i.e., the carboxylic acids (C1-C8), ethanol, and lactic acid concentrations in COD (C_p) with respect to the tCOD fed before obtaining these concentrations and corrected for their presence in the feed (Equation (5-3)).

$$HRT_{(i)} = \frac{V_{reactor} \times \left(t_{(i+1)} - t_{(i)}\right)}{V_{feed(i)}} = \frac{V_{reactor}}{Q_{(i)}} \; [d] \quad (5\text{-}1)$$

$$OLR_{(i)} = \frac{C_{feed(i)}}{HRT_{(i)}} \; [gCOD\, L^{-1} d^{-1}] \quad (5\text{-}2)$$

$$Y_{p(i)} = \frac{C_{p(i),effluent} - C_{p(i-1),feed}}{C_{feed(i-1)}} [\%] \quad (5\text{-}3)$$

where t represents the day of reactor operation, and $t_{(i+1)} - t_{(i)}$ is the time between feeding points, i.e., the time of one semi-continuous fermentation cycle. The instantaneous organic load (IOL) was calculated to distinguish between the average OLR and the organic load applied at the moment of feeding (Equation (5-4)).

$$IOL_{(i)} = \frac{C_{feed(i)} \times V_{feed(i)}}{V_{reactor}} \; [gCOD\, L^{-1}] \quad (5\text{-}4)$$

5.1.2.3. Chemical analysis

Samples of reactor effluent were collected to analyse liquid fermentation products twice a week and for solids and COD once a week. Samples were stored at -20 °C, except for solid analysis. During cycle studies, liquid samples were taken throughout the fermentation cycle, i.e., in between feeding,via the outlet port, and immediately diluted, filtered and frozen at -20 °C following analysis protocol. C1-C5 carboxylic acids, ethanol, lactic acid, and sugars in the FW samples were measured by high pressure liquid chromatography with a refractive index detector (HPLC-RID, Shimadzu, Kyoto, Japan) equipped with Aminex® HPX-87H column (Bio-Rad, CA, USA) as in Coma, et al. [22] with the oven temperature adjusted to 65 °C. MCCA (C6-C8) were measured by gas chromatography with a flame ionization detector (GC-FID, Shimadzu, Kyoto, Japan) on a DB-FFAP (30m×0.25mm×0.25µm) column (Agilent Technologies, Santa Clara, CA, USA) following a sample preparation and method adapted from Manni and Caron [23], as published previously [14].

Solids content was determined using fresh samples according to Standard Methods 2540 D and E [24]. Chemical oxidation demand (COD) was measured after diluting samples into the measuring range by colorimetric test kits (Cat. 2125915, Hach, Düsseldorf, Germany) following Standard Method 5220 D before and after filtration (0.45 µm) for total and soluble COD, respectively [24].

5.1.2.4. 16s rRNA amplicon sequencing

To characterise the microbial community composition biomass samples were taken and immediately stored (-80 °C) at the start of reactor operation, and then at regular intervals from the feedstock and reactor effluent. This amounted to 6 samples for the feedstock and 17 samples for each reactor. DNA was extracted using the FastDNA Spin kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocol with a slight modification in the homogenization step. Sludge homogenization using a FastPrep™ FP120 instrument (MP Biomedicals, Santa Ana, CA, USA) was increased from 1 × 40 s to 4 × 40 s at 6 m s^{-1} to increase the DNA yield [25]. The extracted DNA was purified using the DNA Clean and Concentrator™-10 purification kit (Zymo Research, Irvine, CA, USA). The purified DNA was quantified and the quality checked with a Qubit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and NanoDrop™ 2000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA), respectively. The DNA samples were stored (- 80 °C) prior to preparation of the 16S rRNA gene region V4 sequencing amplicon library. To cover both archaeal and bacterial domains, PCR amplifications targeting the v4 region were done with the primer pair of 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3') [26]. The PCR program involved an initial denaturation for 2 min at 95 °C followed by 25 cycles of amplification (95 °C for 20 s, 55 °C for 15 s, 72 °C for 1 min) and a final elongation for 10 min at 72 °C. A quality check was performed for size and success of PCR with TapeStation (Agilent Technologies, Santa Clara, CA, USA). PCR products were purified and quantified using Agencourt Ampure XP Beads (Beckman Coulter, Brea, CA, USA) similar to the standard protocol with a modified 1:0.8 bead ratio, and Qubit™ dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA), respectively. The PCR products were submitted to the SCELSE sequencing facility (Singapore) for indexing and sequencing (paired-end, 2×300 bp) using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Forward and reverse reads were trimmed (i.e., at 240 and 220 nucleotides for forward and reverse reads, respectively, as this was when average quality dropped below a Phred score of 20). Reads were then dereplicated and merged to construct an amplicon sequence variant (ASV) table following the DADA2 pipeline (1.16) [27]. The reads were clustered and taxonomy assigned at 97% identity using the SILVA v137 database [28].

5.1.2.5. Statistical analysis and data visualisation

Statistical analysis was performed in R version 4.0.3 through the RStudio IDE using functions in the R Stats Package (v3.6.2) and additional packages described below [29].

5.1.2.5.1. Comparison of reactor performance

Reactor performance was evaluated using the net product yields and concentrations of liquid fermentation compounds, i.e., carboxylic acids, ethanol and lactic acid, and the pH of reactor effluent and NaOH dosing. Differences between duplicate reactors and the effect of OLR or feeding pattern were determined with a Wilcoxon-Mann-Whitney test [30]. This test was chosen as normal distribution of pairwise differences was not guaranteed for all fermentation compounds. This test does assume independence of all samples taken at different time points in the same reactors, which is an inherent limitation of the approach. The resulting p-values were corrected for multiple comparisons using a false discovery rate (FDR) of 5% [31]. The figures used to visualise statistical differences were made using the R package *ggplot2* (v3.3.2) and its accompanying sub-packages [32].

5.1.2.5.2. Microbial community evaluation

To evaluate and compare microbial community dynamics, the 16S rRNA sequencing results were analysed and visualised using the *vegan* (v2.5-6) and *ampvis2* (v2.6.6) packages in R [33, 34]. ASV that were not classified as Bacteria or Archaea were removed. The resulting ASV abundance data had an even sequence depth across samples (all within 66k and 104k) and was rarefied to the lowest sequence read (rarefaction curves in Figure S5-2) and normalised [35]. Effective alpha-diversity, i.e., 1st and 2nd Hill number (^{1}D and ^{2}D), were calculated using the *amp_alphadiv* function to obtain Simpson and Shannon indices and converted according to reference to compare community structures [36-38]. Effective alpha-diversity indices are preferred over more traditional ecological indices, e.g., Shannon or Simpson index, and measures of richness since they are more robust, intuitive, and comparable among different studies [38, 39]. The relative abundance data was analysed using heatmap visualisations, indicator species analysis and ordination plots. Heatmaps were constructed using the *amp_heatmap* function. Indicator species analysis with correlation indices was performed to determine the ecological preference of ASVs among bi-weekly or daily fed reactors using the *multipatt* function with “r.g.” from the *indicspecies* package (v1.7.8, 2020) [40]. The relative abundance data was Hellinger transformed and analysed using a Non-Metric Multidimensional Scaling (NMDS) plot on the Bray-Curtis dissimilarity matrix using the *metaMDS* function. The Hellinger transformation is preferred for species abundance data as it corrects for low counts with many zeros by giving them a low weight [41]. The NMDS plots were visualised using the *ggplot2* and *ggordiplots* package to include ellipses based on standard deviation (95% confidence interval) to group per

feeding pattern [32, 42]. Values of stress below 0.2 on the NMDS plots are acceptable [41]. Permutational multivariate analysis of variance (PERMANOVA) tests were performed using the *adonis* function (9,999 permutations) on the same data used to generate the NMDS plots to evaluate whether differences in community structure depending on feeding pattern were significant [43]. A permutation test for homogeneity of multivariate dispersion (PERMDISP, 9,999 permutations) tests was performed to check this assumption that is required for the PERMANOVA test [34, 44]. The p-values from the multivariate test were corrected for multiple comparisons using a FDR of 5% [31].

5.1.2.5.3. Correlation analysis

The Spearman's rank correlation coefficient (r_S) was determined between different products and between the relative abundance of specific ASV and product yield (R *Stats* package (v3.6.2)). Matrix of correlation coefficients were made for reactor performance and visualised using the *Hmisc* (v4.4-1) and *corrplot* (v0.84) packages [45, 46]. Data during reactor start-up and outliers were excluded. Outliers in operation were caused by 8 disrupted feeding events (less than 5% of total feeding events) due to pumping faults. The p-values from the correlation analysis were corrected for multiple comparisons using a FDR of 5% [31].

5.1.3. Results and discussion

5.1.3.1. Co-fermentation of soy wastewater and food waste selects for even-chain carboxylates, ethanol and lactic acid

Four sCSTR were fed with a mixture of FW and Soy as described in Section 5.1.2.2. Although the effluent composition varied over time (Figure S5-3) and in relation to feeding pattern, the main products of fermentation were ethanol, lactic acid and even-chain MCCA, i.e., mostly n-caproic (C6) and some n-caprylic (C8) acid (Figure 5-1). During the first 70 days of reactor operation the average tCOD of the feedstock was 132 ± 19 gCOD L^{-1} resulting in an average OLR of 13 ± 2 gCOD L^{-1} d^{-1}. Half of the tCOD from the feedstock (51 ± 9 %) was metabolised into liquid products (i.e., carboxylic acids, ethanol or lactic acid) in all reactors. To improve yields and reduce lactic acid accumulation, on Day 70 the OLR was lowered by decreasing the FW/Soy ratio in the feedstock, giving an average tCOD content of 90 ± 19 gCOD L^{-1} and, thus an average OLR of 9 ± 2 gCOD L^{-1} d^{-1}. The overall product yield (i.e., soluble products/feedstock COD) significantly increased and the amount of feedstock converted to liquid products increased to 67 ± 13 % ($p<0.0001$) (Figure S5-4).

The total yield of shorter chain carboxylates, i.e., acetic (C2) and n-butyric (C4) acid, generally remained below 20% for all operating conditions. The MCCA were produced at average concentrations of 16 ± 6 gCOD L^{-1} for C6 and 8 ± 3 gCOD L^{-1} C8, which are in a

similar range to previous reports on semi-continuous, single-stage MCCA production from FW fermentation without supplementation of external electron donors [12, 14]. Thus, co-fermentation of FW and Soy lead to MCCA production in a single-stage reactor without physically separating primary and secondary fermentation. Considering the residual concentrations of primary fermentation products, chain elongation could potentially be further optimised by, for instance, increasing retention times or applying in-line product extraction. A FW feedstock requires a long HRT to allow for sufficient hydrolysis and time for secondary fermentation to occur [10, 21]. In-line product extraction could potentially help to overcome limits imposed by thermodynamics or toxicity due to product accumulation [47, 48].

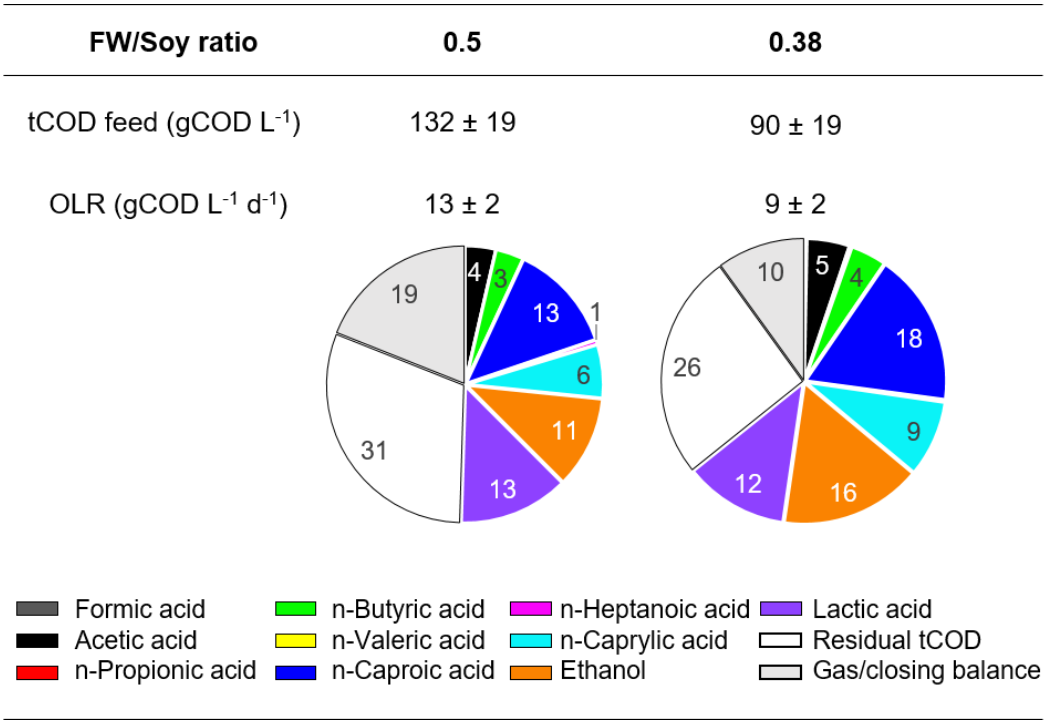


Figure 5-1 *Distribution of organics in the effluent at different feed ratios of food waste (FW) to soybean wastewater (Soy). Results are averaged over the four reactors. The residual tCOD is determined by measurement of the tCOD of the effluent. The tCOD that could not be accounted for was attributed to loss of organics and hydrogen in the gas phase.*

Odd-chain carboxylic acids were detected in very low quantities. n-Propionic acid (C3) was only present at concentrations above 1 gCOD L^{-1} during the start-up phase, and longer odd-chained carboxylic acids, i.e., n-valeric (C5) and n-heptanoic (C7) acid were washed out (Figure S5-5). C5 and C7 are products from chain elongation of C3 [49]. C3 is usually a common FW fermentation product obtained via lactate reduction or the reversible transcarboxylase cycle in e.g., *Propionibacterium* or *Bifidobacterium* spp. [5]. Accumulation of C3 in FW fermentation has been shown to compromise chain elongation performance at high lactic acid influent rates and when pH is maintained above 6 [50, 51]. Even though pH averaged between 5.8 and 6.2, and lactic acid concentration peaked occasionally in the

reactors, i.e., up to 43 gCOD L^{-1}, there was no net production of C3. This could be due to the relatively long HRT (10.5 days), which caused the actual lactic acid accumulation rate to never exceed 5 gCOD L^{-1} d^{-1}. This was below the lactate loading rates found necessary for C3-production to compete with chain elongation by Kucek, et al. [50], who used a clean, synthetic feed and operated at an HRT of <2 days. Thus, due to the long HRT required for chain elongation in FW fermentation, lactic acid accumulation rates were too low to allow for C3 production, which aids product selectivity.

5.1.3.2. Longer batch cycles reduced acidogenic lactic acid accumulation

From the four reactors operated, two reactors were fed bi-weekly, i.e., every 3.5 days ($sCSTR_{BW}$), and two reactors were fed daily ($sCSTR_{D}$). Thus, while the average OLR was similar for all reactors, the instantaneous organic load (IOL), i.e., organic load applied at feeding events, was 3.5 times higher when feeding twice a week compared to feeding daily. All four reactors presented a fluctuating output composition regardless of the feeding pattern, especially with respect to lactic acid content (Figure 5-2). However, despite this instability in product outcome, some clear observations could be made.

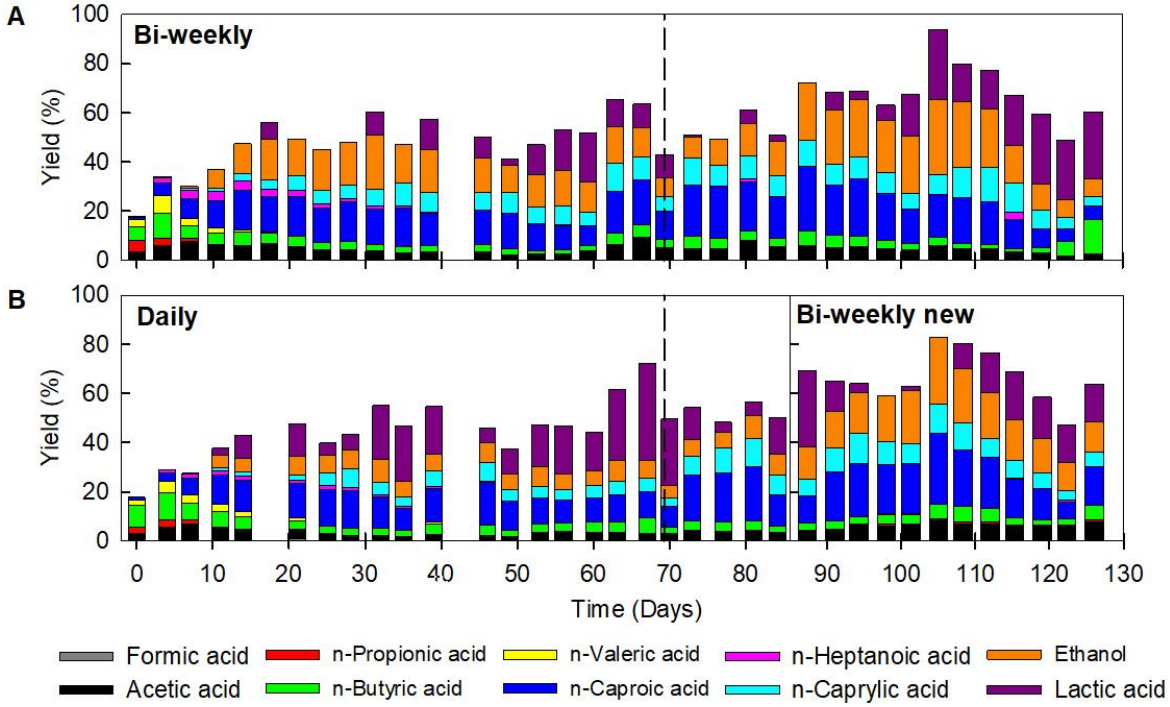


Figure 5-2 *Product yields from co-fermentation of food waste and soybean soaking wastewater. Yields are averaged over duplicate reactors fed twice a week (A, $sCSTR_{BW}$), or daily and switched to bi-weekly feeding after 8 HRT of operation (B, $sCSTR_{D/BW\text{-}new}$). The dashed lines represent the day on which the OLR was reduced from 13 to 9 ± 2 gCOD L^{-1}. Product yields for the individual reactors are available in Figure S5-3.*

5.1.3.2.1. Daily feeding led to more lactic acid in the effluent

The total conversion of organics to liquid fermentation products was similar for all reactors regardless of feeding pattern (i.e., 52 ± 7% for $sCSTR_{BW}$ and 51 ± 8% for $sCSTR_{D}$). However, yields of specific compounds differed significantly between bi-weekly and daily

fed reactors according to statistical tests (Figure 5-3 A). The average yield of lactic acid in the daily fed reactors ($sCSTR_D$: Y_{LA} = 16 ± 12 %) was nearly double than in the bi-weekly fed ones ($sCSTR_{BW}$: Y_{LA} = 7 ± 8 %) ($p<0.001$). This is in line with the more variable and higher lactic acid concentrations observed in the effluent of $sCSTR_D$ as discussed later. In contrast, the ethanol, C2 and C8 yields were nearly halved for daily fed reactors ($sCSTR_D$: $Y_{ethanol}$ = 7 ± 2%, Y_{C2} = 3 ± 1%, Y_{C8} = 6 ± 3%) compared to bi-weekly feeding ($sCSTR_{BW}$: $Y_{ethanol}$ = 14 ± 5%, Y_{C2} = 5 ± 2%, Y_{C8} = 8 ± 2) ($p<0.01$). The increased C8 yields in the bi-weekly fed reactors could result from the increased ethanol concentrations, as ethanol-based chain elongation has been linked before to C8 production rather than lactate-based chain elongation [52].

After 8 HRTs, the two $sCSTR_D$ systems were switched to a bi-weekly feeding pattern (and labelled $sCSTR_{BW\text{-}new}$) to confirm whether the feeding pattern impacted fermentation performance. The resulting lactic and ethanol yields of $sCSTR_{BW\text{-}new}$ were similar to the $sCSTR_{BW}$ systems (Figure 5-3 B). Thus, while overall conversion was not affected by the feeding pattern, more organics were fermented to lactic acid instead of C2 or ethanol when feeding daily compared to bi-weekly. To better understand the potential impact of feeding pattern, other aspects of reactor performance, namely addition of pH-correcting chemicals, repeatability, and microbial community composition, were analysed.

5.1.3.2.2. Reducing the need for pH correction by feeding bi-weekly

Lactic acid-type fermentation is characterised by rapid acidification and the potential to ferment at a pH as low as 3.5 [53, 54]. By contrast, chain elongation in FW fermentation has a pH-neutralising effect since lactic acid and VFA are net consumed [13]. The pH in all reactors was controlled at a minimum of 5.5. This is sufficiently high for VFA fermentation, and prevents chain elongation from being hindered by acid inhibition [10, 52]. The amount of NaOH dosed to maintain a minimum pH reflected the level of acidification of the fermentation. The pH in the bi-weekly fed reactors was regularly higher and averaged 6.2 ± 0.5, in line with the pH-increasing effect of chain elongation and the slightly higher C8 yields. The fermentation in the daily-fed reactors was more acidogenic with the effluent having a lower average pH at 5.8 ± 0.4. The average NaOH dosing for the two $sCSTR_{BW}$ reactors (1.1 ± 0.7 g $L^{-1}d^{-1}$) was only half of that for the two $sCSTR_D$ systems (2 ± 2 g $L^{-1}d^{-1}$) ($p<0.05$) (Figure 5-4 A). This difference disappeared when all reactors were operated with a bi-weekly feeding pattern, i.e., when the two $sCSTR_D$ were switched to a bi-weekly feeding pattern ($sCSTR_{BW\text{-}new}$) (Figure 5-4 B). The addition of pH-correcting chemicals increases the operational and environmental cost of FW fermentation for MCCA production [55]. Thus, by operating with bi-weekly feeding, less acidogenic lactic acid fermentation occurred, and hence NaOH dosing requirements were reduced, so operating costs were reduced.

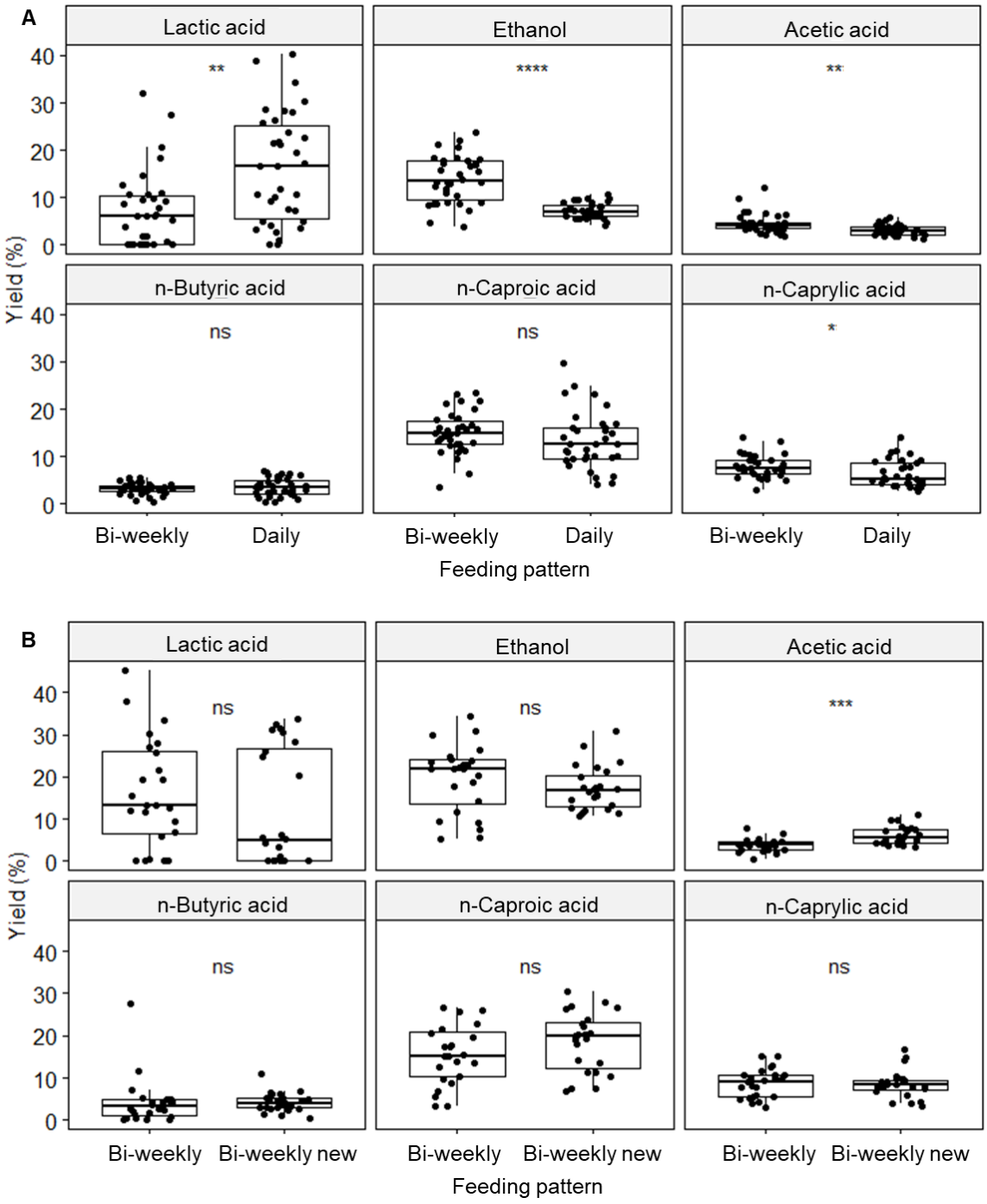


Figure 5-3 *Evaluation of product yields per feeding pattern.* ***A:*** *bi-weekly and daily feeding is compared during the first 8 HRT of operation (excluding start-up).* ***B:*** *yields from after 8 HRT to end of operation are compared between reactors fed bi-weekly and reactors that were previously fed daily and switched to bi-weekly feeding (Bi-weekly new). Symbols "ns", "**", "***" and "****" represent p-values of >0.5 (not significant), <0.01, <0.001 and <0.0001, respectively, resulting from a Wilcoxon-Mann-Whitney test. A similar analysis is available on product concentrations (Figure S5-6).*

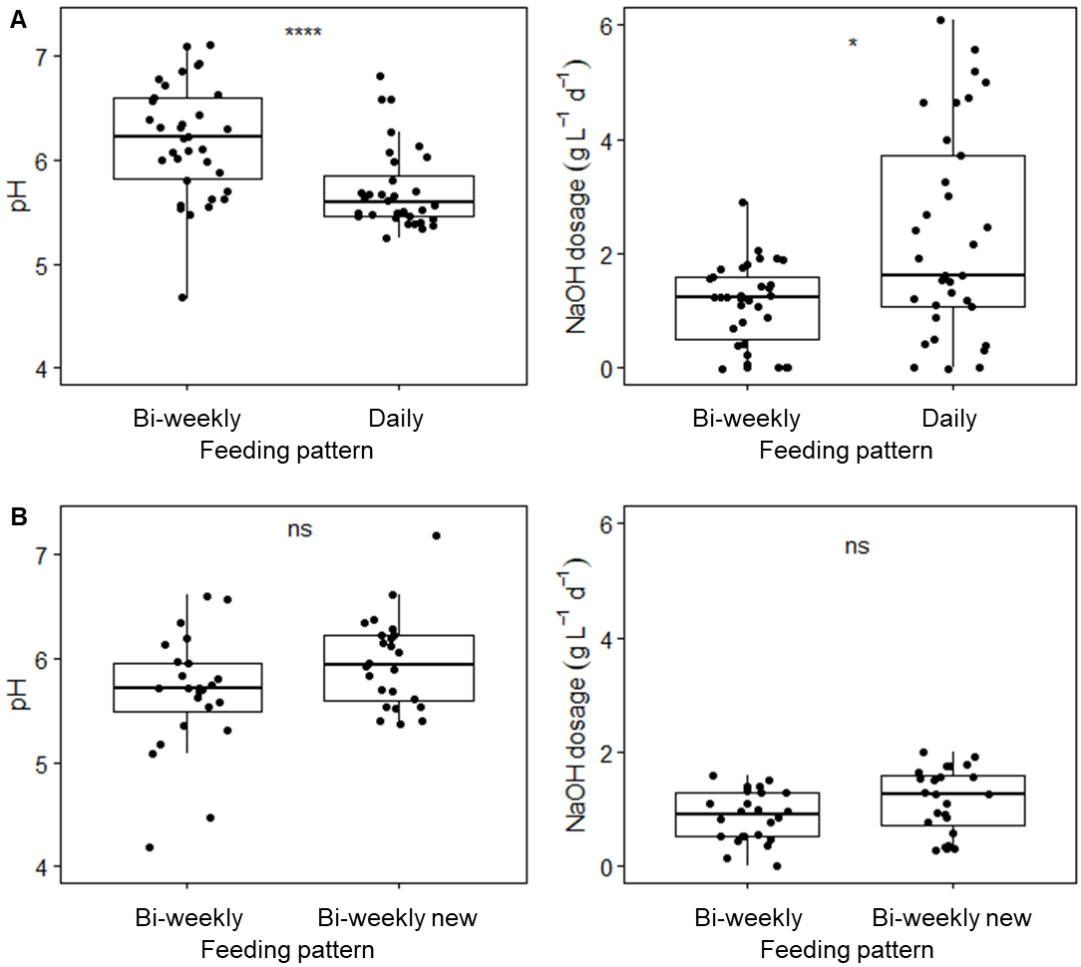


Figure 5-4 *Effluent pH (left) and NaOH dosing requirements (right) to maintain minimum pH (5.5) for the different feeding patterns.* ***A:*** *bi-weekly and daily feeding is compared during the first 8 HRT of operation (excluding start-up).* ***B:*** *yields from after 8 HRT to end of operation are compared between reactors fed bi-weekly and reactors that were previously fed daily and switched to bi-weekly feeding (Bi-weekly new). Symbols "ns", "*" and "****" represent p-values of >0.05 (not significant), <0.05 and < 0.0001, respectively.*

5.1.3.2.3. Bi-weekly feeding improved performance stability

In terms of repeatability, the duplicates of the bi-weekly fed reactors ($sCSTR_{BW}$ 1 and 2) had similar overall reactor performance (Figure 5-5 A). By contrast, the reactors fed daily ($sCSTR_D$ 1 and 2) had greater differences in performance when compared to each other. $sCSTR_D$ 2 had significantly ($p<0.01$) lower yields for C4 (2 ± 2 %) and C6 (3 ± 6 %) but higher C8 (7 ± 2 %) compared to $sCSTR_D$ 1 (Y_{C4} = 5 ± 1 %, Y_{C6} = 15 ± 4 %, Y_{C8} = 4.1 ± 0.9 %) (Figure 5-5 B). In addition, the two $sCSTR_D$ systems showed more fluctuation in product outcome and higher peaks in lactic acid yield compared to $sCSTR_{BW}$ (Figure 5-6). Thus, the performance of the bi-weekly fed reactors showed better control of product outcome with less lactic acid production than the daily-fed reactors.

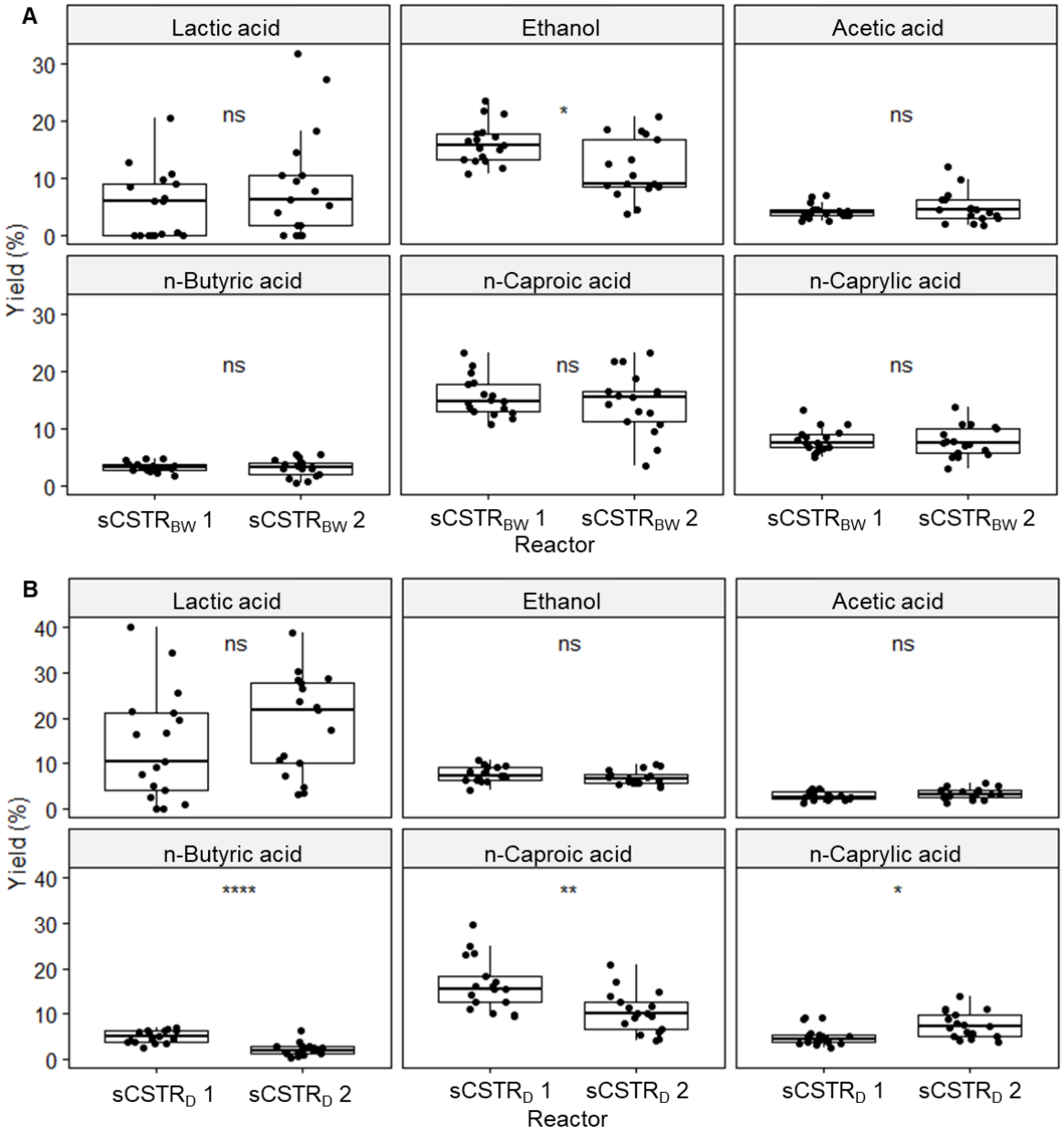


Figure 5-5 *Performance of duplicate reactors during the first 8 HRT of operation (excluding start-up) compared using a Wilcoxon-Mann-Whitney rank sum test.* ***A****: the duplicate reactors that were fed bi-weekly (sCSTR$_{BW}$ 1 and 2).* ***B****: the duplicate reactors that were fed daily (sCSTR$_{D}$ 1 and 2). Comparative analysis was also performed on data after 8 HRT of operation until end (Figure S5-7). Symbols "ns", "****" and "*****" represent p-values of > 0.5, < 0.001 and < 0.0001, respectively.*

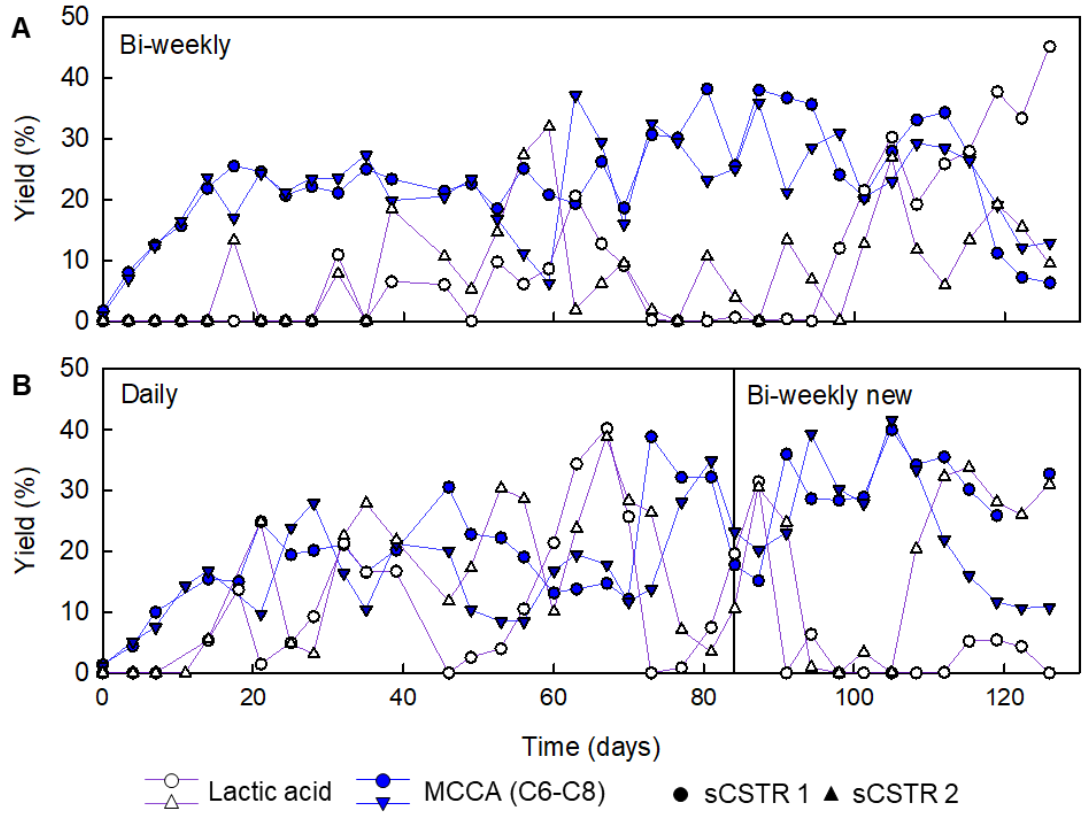


Figure 5-6 *The net lactic acid and MCCA yields fluctuated throughout operation in the four reactors.* ***A****: reactors fed bi-weekly.* ***B****: reactors fed daily and switched to bi-weekly later as indicated on top of the graph. Symbols represent reactor system and colours indicate compound.*

5.1.3.2.4. Lactic acid and MCCA yields were negatively correlated

Regardless of the feeding pattern, lactic acid concentrations in the effluent fluctuated over time from 0 gCOD L^{-1} to peaks as high as 43 gCOD L^{-1}. The presence of lactic acid in the effluent appeared to change in inversely proportion to MCCA concentration (Figure 5-6). Correlation analysis confirmed a negative correlation between lactic acid and C6 (r_S = -0.76, p<0.001) (Figure S5-8). With increased lactic acid yield the medium acidified and more NaOH was required to maintain the minimum pH (r_S = 0.54, p<0.001). By contrast, with increased C6 and C8 yields less NaOH had to be dosed (r_S = -0.48 and r_S = -0.36, respectively, p<0.001). This is because lactic acid production is acidogenic whereas chain elongation increases pH. Similar trends were seen for different reactors, OLR or feeding patterns (Figure S5-9). Furthermore, the yields of carboxylic acids and ethanol were weakly correlated to each other (0.31 < r_S < 0.56, p<0.01). Thus, regarding reactor performance two types of fermentation alternated. On one hand acidogenic lactic acid-type fermentation occurred and on the other hand, a mixed acid and ethanol fermentation with chain elongation took place. Thus, a balance between two net metabolic pathways was observed. A bi-weekly feeding pattern seemed to stabilise this balance more towards ethanol and chain elongation compared to a daily feeding.

5.1.3.3. Microbial community dynamics reflected reactor performance

5.1.3.3.1. Development of a specialised microbial community for acidogenic food waste fermentation

To better understand the processes governing reactor performance, the microbial community composition of the inoculum, the feedstock and reactors was analysed. The microbial community in the inoculum was distinct from that in the feedstock or reactors (Figure 5-7), and based on the Hill numbers ^{1}D and ^{2}D, the alpha-diversity of the inoculum was 10 times higher (Table 5-1). The inoculum was obtained from a full-scale sewage sludge anaerobic digester. The diverse bacterial community is typical for full-scale AD of sewage sludge and co-digestion at phylum level for Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Chloroflexi and Synergistetes, in combination with a lower relative abundance of Euryarchaeota [56, 57].

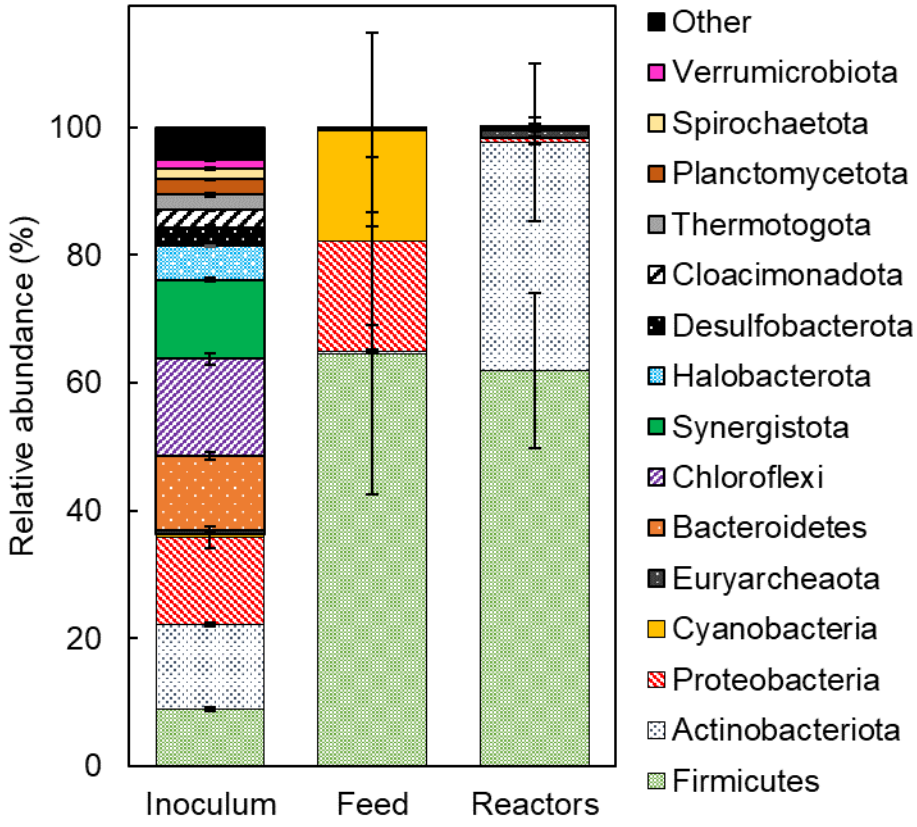


Figure 5-7 *Microbial community composition at the phylum level in the inoculum, feedstock and reactors averaged over 4, 6 and 64 samples, respectively.*

The feedstock harboured a less diverse community than the inoculum (Table 5-1). It contained predominantly Firmicutes (65 ± 22%), Cyanobacteria (18 ± 15%) and Proteobacteria (17 ± 13%). The presence of Cyanobacteria can be attributed to their use as food in certain cuisine [58].

Table 5-1 *Alpha-diversity indices based for the microbial community in the inoculum, feedstock and reactors.*

Index	Inoculum	Feed	Reactors
Number of samples	4	6	64
Observed ASVs	748 ± 28	144 ± 28	80 ± 14
^{1}D (exponent Shannon)	76 ± 4	8 ± 3	6 ± 3
^{2}D (inverse Simpson)	182 ± 7	17 ± 5	11 ± 4

Proteobacteria included members of *Pseudomonas* spp. (3 ± 2%) and *Acinetobacter* spp. (6 ± 8%) which have been linked to potential pathogen development in food waste [59]. The Firmicutes were mostly lactic acid bacteria typical of fermented foods or food spoilage, such as *Weissella* spp. (25 ± 15%), *Lactobacillus* spp. (10 ± 17%), *Leuconostoc* spp. (14 ± 4%) and *Lactococcus* spp. (8 ± 8%) [60, 61]. Their presence resulted in acidification of the feedstock within a couple of days of storage. Within three days of storage at 4 °C, lactic acid fermentation occurred (from 0.19 ± 0.01 g L^{-1} to 1.84 ± 0.02 g L^{-1}), resulting in the pH of the feedstock dropping from 6.24 ± 0.02 to 4.49 ± 0.02 (Table S5-2). Changes of feedstock quality during storage is an important consideration, especially for a highly fermentable feedstock such as FW [62]. It can affect yields in AD, hydrogen fermentation, and chain elongation [63-65].

The reactors showed the least diverse and rich communities, with the formation of a more specialised community of Firmicutes (62 ± 12%) and Actinobacteria (36 ± 12%). This is similar to previous work directing FW fermentation towards acidogenic fermentation and chain elongation products [14].

5.1.3.3.2. Communities per feeding pattern align with fermentation outcome

To evaluate the influence of feeding pattern on microbial community composition, ordination plots were made, and statistical tests were run, to compare the community in reactors fed bi-weekly with those in daily fed reactors. It was also possible to test whether communities converged when daily fed reactors were switched to bi-weekly feeding (bi-weekly new) in a second operational phase. Significantly different communities were found for different feeding patterns during the first operational phase (PERMANOVA, $p < 0.001$, Table S5-4) (Figure 5-8 A). In the second operational phase, when all reactors were operated with a bi-weekly feeding pattern, the communities in the reactors previously fed daily were more similar to those kept on a bi-weekly feeding approach (see the overlap of ellipses in Figure 5-8 B). However, communities still differed, albeit at a lower significance (PERMANOVA, $p = 0.0024$, Table S5-4). Thus, feeding pattern had some influence on microbial community composition, however many other factors such as operating time, feedstock composition or even minor disturbances could have affected community dynamics. Determining what

governs the link between operation and the resultant microbial community composition and structure remains a challenging and compelling topic in the field of microbiome research and engineering [39, 66].

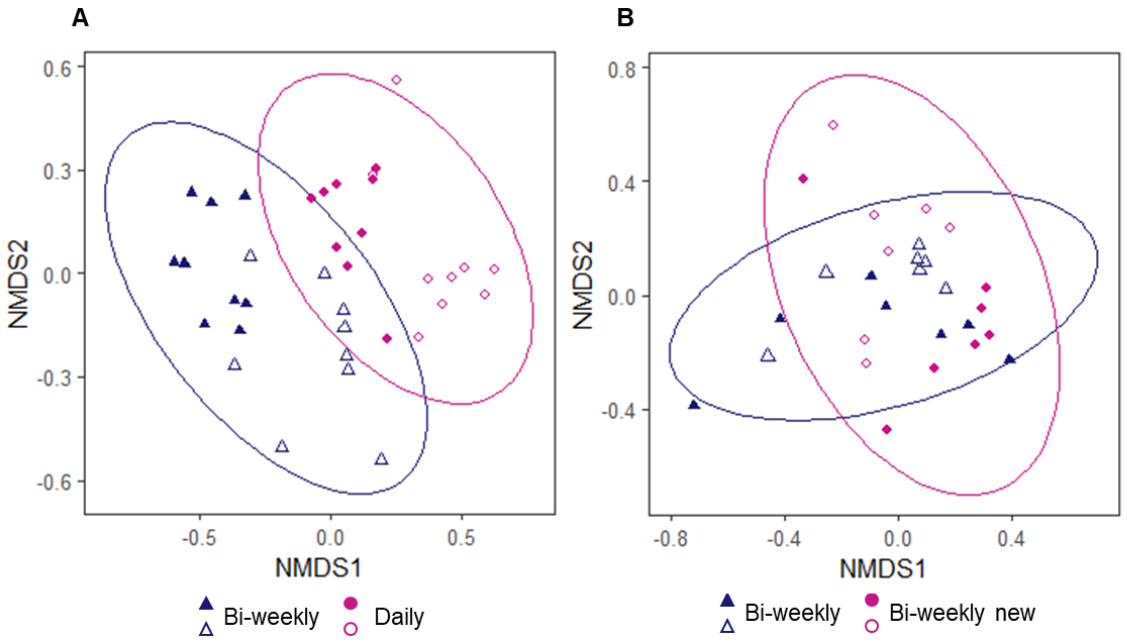


Figure 5-8 *Non-metric multidimensional scaling analysis (NMDS) of Hellinger transformed relative ASV abundance data using the Bray-Curtis dissimilarity matrix (stress = 0.11). Each point represent the community in a specific sample. The closer the sample points on the plot, the greater the similarities between the communities in the samples.* ***A:*** *The community in bi-weekly and daily fed reactors (36 samples from the first 8 HRT of operation excl. start-up).* ***B:*** *Reactors fed bi-weekly and reactors that were previously fed daily but switched to bi-weekly feeding (Bi-weekly new). Filled symbols show different duplicates: $sCSTR_{BW}$ 1 ▲; $sCSTR_{BW}$ 2 △; $sCSTR_{D/BW\text{-}new}$ 1 ●; $sCSTR_{D/BW\text{-}new}$ 2 ○).*

A heatmap was constructed to visualise which genera had the highest relative abundance for each feeding pattern (Figure 5-9). The daily fed reactors showed a higher relative abundance of the lactic acid bacteria *Lactobacillus* spp. (47 ± 12 %) and *Olsenella* spp. (28.5 ± 14 %) compared to the bi-weekly fed reactors (34.4 ± 13 and 20.3 ± 14 %, respectively). Since the genus of *Lactobacillus* is very large and diverse, the most abundant ASVs that linked to this genus were further analysed (Figure 5-10). It was noted that *Lactobacillus* ASV 1 was generally more abundant for bi-weekly fed reactors whereas *Lactobacillus* ASV 2 had a much higher abundance in the daily fed systems. An indicator species analysis comparing the different feeding patterns confirmed that ASV 2 was indicative for the daily fed reactors ($p<0.001$) (Table S5-5). Running the sequence of ASV 2 through a BLAST search gave high similarity (ID 100%) to strains of *L. amylovorus* and *L. acidophilus*, thus ASV 2 was likely to have a similar homolactic metabolism [67]. The increased presence of homolactic bacteria in the daily fed reactors is consistent with the reactor performance of higher lactic acid yield and NaOH dosing requirements.

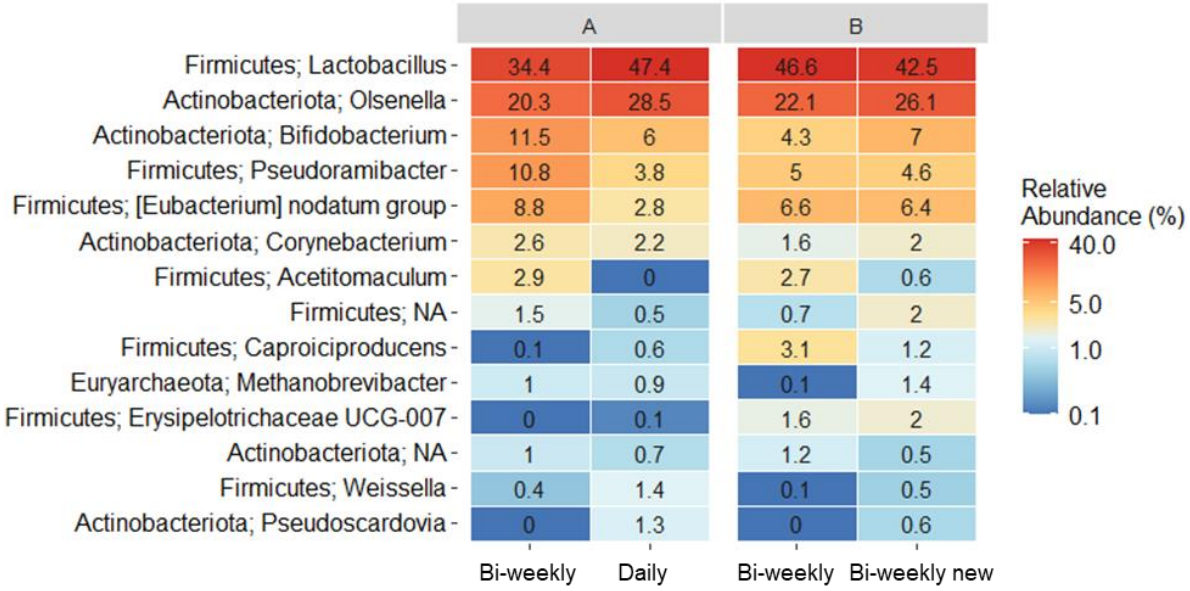


Figure 5-9 *Heatmap of the relative abundance of the genera present in (**A**) bi-weekly and daily fed reactors (from the first 8 HRT of operation excl. start-up) and (**B**) in the reactors fed bi-weekly and reactors that were previously fed daily but switched to bi-weekly feeding (Bi-weekly new). Only genera with an average relative abundance >1% are shown.*

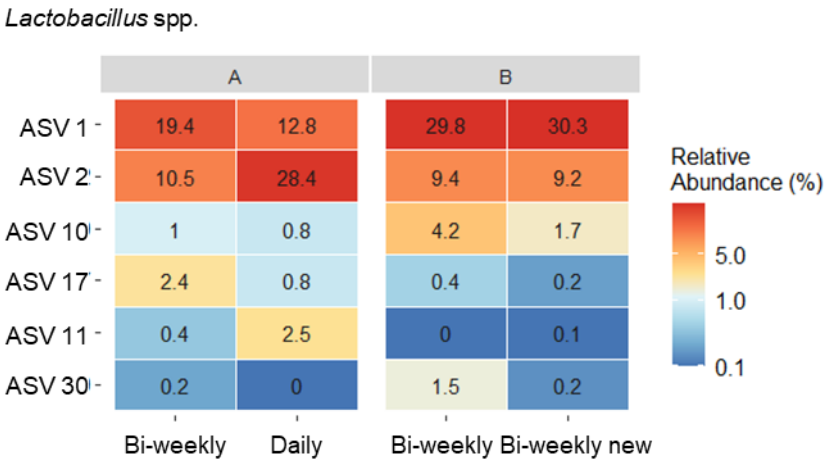


Figure 5-10 *Heatmap of the ASV linked to Lactobacillus spp. in (**A**) bi-weekly and daily fed reactors (from the first 8 HRT of operation excl. start-up) and (**B**) the reactors fed bi-weekly and reactors that were previously fed daily but switched to bi-weekly feeding (Bi-weekly new). Only ASV with an average relative abundance >1% are shown.*

The indicator species analysis also associated ASVs with a relative abundance >1% with the bi-weekly feeding strategy as ASV 8 (*Olsenella* spp.), ASV 6 (*Pseudoramibacter* spp.) and ASV 7 (*[Eubacterium] nodatum group* spp.) (Table S5-5). The heatmap visually confirmed that *Pseudoramibacter* spp. and species belonging to *[Eubacterium] nodatum* group were present at higher relative abundance in the bi-weekly fed reactors compared to daily fed systems (Figure 5-9). *Pseudoramibacter* spp. have been linked with lactic acid-based chain elongation [68]. Species belonging to *[Eubacterium] nodatum* group have been linked to n-butyrate production and were present in chain elongation microbiomes

processing FW [14, 69-71]. Thus, bi-weekly fed reactors evolved a microbial community comprising genera typically linked to chain elongation by contrast to the daily fed reactors.

5.1.3.3.3. Microbial community changed in line with oscillations in effluent composition

The microbial community composition fluctuated over time, as observed with reactor performance, regardless of feeding pattern (Figure 5-11). Therefore, an additional correlation analysis was performed with 15 of the most abundant ASVs (Table S5-6). Several ASVs were found to have strong correlations with either lactic acid, ethanol, C2 or MCCA. Different ASV in the same genera showed opposite correlations, such as the most abundant ASVs, ASV 1 and ASV 2. Both were classified as part of the *Lactobacillus* genus. ASV 2 was found to be an indicator species for the daily fed reactors with likely a homolactic fermentation metabolism. Correlation analysis confirmed that it was positively correlated with lactic acid ($p<0.0001$, $r_S = 0.52$), and negatively with ethanol, C2 and C6 yields ($p<0.0001$, $r_{S,ethanol} = -0.77$, $r_{S,C6} = -0.47$) (Figure S5-11). In contrast, ASV 1 had a strong positive correlation with ethanol, C2 and C6 yields ($p<0.01$, $r_{S,ethanol} = 0.72$, $r_{S,C2} = 0.49$, $r_{S,C6} = 0.41$). ASV 1 is therefore more likely to code for a heterolactic fermentation metabolism which produces ethanol and acetic acid alongside lactic acid, and thereby providing all the necessary precursors for chain elongation to C6.

The highest positive correlation with chain elongation products was found for ASV 6, belonging to *Pseudoramibacter* spp. with C6 yield ($p<0.0001$, $r_S = 0.62$) and for ASV 7 with C8 yield, *[Eubacterium] nodatum group* spp. ($p<0.0001$, $r_S = 0.48$) (Table S5-6). Both of these were characteristic species for the bi-weekly fed reactors and they are known to perform lactic acid-based chain elongation (Section 5.1.3.3.2.). It is interesting to note that the *Pseudoramibacter* ASV 6 was strongly negatively correlated with lactic acid ($p<0.0001$, $r_S = -0.76$). Overall, the differences observed in the reactor performance, i.e., favouring of either chain elongation or lactic acid production, was reflected in the microbial community composition. However, what factors dictate the switch between homolactic fermenters and a community performing heterolactic and ethanol-acetate type fermentation followed by chain elongation?

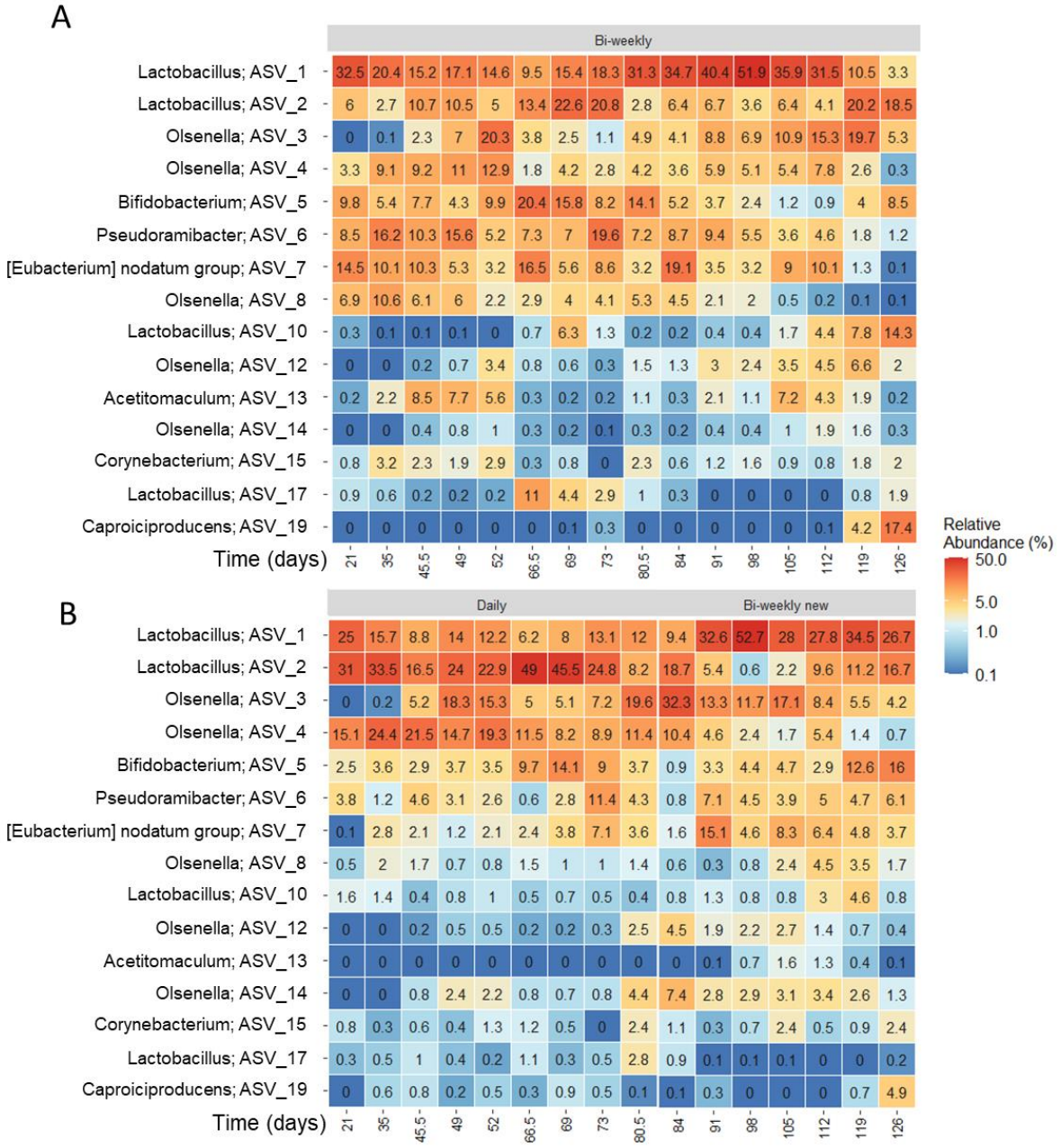


Figure 5-11 *Heatmap of the relative abundance of the ASVs with their corresponding classification at the genus level over time. Values are averaged over duplicates in (**A**) the bi-weekly fed reactors and (**B**) the reactors fed daily and switched to bi-weekly feeding after 8 HRT as indicated on top of the graph. Only genera with an average relative abundance >1% are shown. Heatmap of the relative abundances averaged at the genus level are provided in Figure S5-10.*

5.1.3.4. Unravelling the balance between homolactic fermentation and chain elongation via cycle studies

To gain an insight into the bioconversion processes taking place between feeding events, cycle studies were performed. Here, the reactor contents were sampled at regular intervals over 3.5 days, i.e., the time interval between feeding for the bi-weekly fed reactors. The results of the cycle studies are summarised in supplementary information (Figure S5-12 and S5-13). During the cycle studies, the C6, lactic acid and ethanol concentrations changed the most, whereas concentrations of C2 and C4 did not vary more than 2 gCOD L^{-1} from start to end of the sampling intervals.

Regardless of feeding pattern, glucose was detected during the first hours after feeding due to organics solubilisation. In cycle studies where chain elongation products increased, two consecutive fermentation stages could be distinguished after this initial stage of hydrolysis (Figure 5-12 A). In primary fermentation, glucose was consumed, and lactic acid and ethanol were produced. In $sCSTR_{BW}$ 1 glucose was detected in the first 30 hours followed by lactic acid and ethanol peaking at 7.2 gCOD L^{-1} and 16.9 gCOD L^{-1}, respectively. The pH dropped to 5.7 during this primary fermentation stage. This primary fermentation was followed by a second fermentation stage of chain elongation where lactic acid was net consumed, MCCA accumulated up to a maximum of 37 gCOD L^{-1}, and pH increased to 6.5. In addition, ethanol started to decrease slightly after lactic acid reached < 1 gCOD L^{-1} and more MCCA were produced. In the daily fed reactors, the changes in concentration were smaller compared to the bi-weekly fed reactors, and some residual glucose remained present throughout the shorter cycle time of 24 hours (Figure 5-12 B).

The co-existence of these two fermentation pathways is typical for single-stage, lactic acid-based chain elongation systems using a complex feedstock [12, 13]. This is generally characterised by a microbial community structure containing both lactic acid bacteria (LAB), such as *Olsenella, Bifidobacterium* or *Lactobacillus* spp. and chain elongating bacteria, such as *Pseudoramibacter* spp. [52, 69], and as found in this work.

In cycle studies during net lactic acid accumulation, acidogenic lactic acid production occurred, similar to the primary fermentation phase previously discussed. However, after the acidogenic lactic acid production, minimal further fermentation took place. No increase in C6 was observed in any reactors where lactic acid was net produced. During onecycle study glucose increased in the bi-weekly fed reactors up to a peak of 1.8 gCOD L^{-1} in the first 10 to 27 hours. After this glucose decreased to below detection limits while lactic acid rose rapidly to 20.9 gCOD L^{-1} and the pH dropped to 5.7 (Figure S5-12). However, no secondary fermentation took place in the remaining time of the feed cycle.

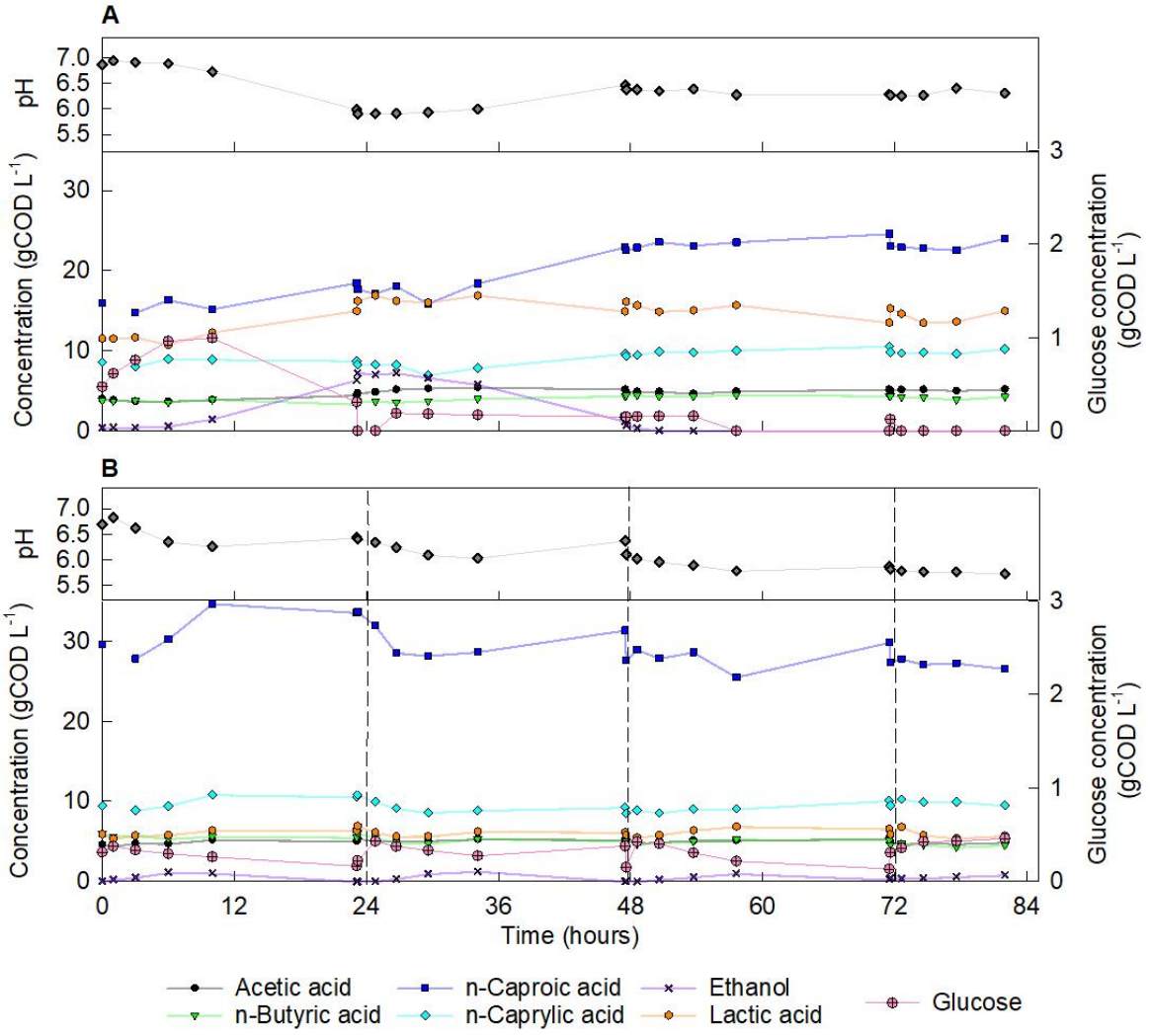


Figure 5-12 *Cycle studies during chain elongation. The pH and concentration profiles of fermentation compounds over 84 hours in (A) a bi-weekly fed reactor (sCSTR$_{BW}$ 1, between Days 73 and 76.5), and (B) a daily fed reactor (sCSTR$_D$ 1, between Days 73 and 76.5). Time 0 corresponds to a sample taken straight after feed addition and the dashed lines indicate feeding events for daily fed reactors. An overview of allcycle studies can be found in SI (Figure S5-12 and S5-13).*

It could be hypothesised that acid inhibition and/or product toxicity hindered further chain elongation. However, pH was controlled above 5.5, i.e., sufficiently high for lactic acid to be converted into VFA or MCCA [8, 72]. Product toxicity can be caused by MCCA accumulation, as their protonated fraction is antimicrobial [73, 74]. However, it is unlikely that this could have prevented secondary fermentation, since for similar amounts of protonated MCCA, both lactic acid accumulation and further chain elongation took place. In sCSTR$_D$ 2, one cycle study resulted in chain elongation and another in lactic acid accumulation, yet the concentration of protonated MCCA was similar in each case, fluctuating between 6 and 12 mM (Figure S5-14). Thus, product toxicity was unlikely to be the decisive factor for secondary fermentation to occur or not.

The difference in net fermentation outcome and community structure could be driven by differences in the availability of easily degradable sugars. The glucose concentrations reached in cycle studies where lactic acid accumulated were double those where chain elongation took place. The presence of sugars has been shown to give LAB a competitive advantage over other fermentative micro-organisms. Rombouts, et al. [75] recently showed that in a microbial community fermenting a complex medium LAB, e.g., *Lactobacillus* spp., will outcompete VFA-producing bacteria. The LAB have a higher biomass-specific glucose uptake rate that provides them with a kinetic advantage despite their metabolism being less energy efficient. Similarly, Park, et al. [76] showed that a *Lactobacillus* sp. would outcompete a *Clostridium* sp. in the presence of glucose (at <1 g L^{-1}) whilst the opposite occurred when feeding galactose. Again glucose provided a selective advantage for the *Lactobacillus* sp.. Certain LAB switch from mixed-acid/heterolactic fermentation to homolactic fermentation at increased growth rates and sugar levels, also known as the anaerobic version of the Crabtree/Warburg effect [77]. The heterolactic-type metabolism is more energetically efficient (i.e., it provides more ATP per glucose) nevertheless, at high sugar levels e.g., at the start of a batch fermentation, LAB opt for a less efficient, but generally quicker homolactic fermentation that can be performed with lower investments in metabolic structures. Various theories have been developed, such as the theory of resource allocation, to explain why this provides LAB with a competitive advantage [78].

Lower concentrations of glucose are more often present in the daily fed sCSTR compared to bi-weekly feeding, providing homolactic LAB with a competitive advantage. During bi-weekly feeding other fermentative bacteria could regain territory once easily fermentable sugars are consumed as the time between feed addition is longer. In one cycle study with $sCSTR_{BW}$ 1, pH started increasing again near the end, with a small decrease in lactic acid concentration (Figure S5-12), indicating that secondary fermentation might have occurred if the fermentation was allowed to continue before feeding. This agrees with the reactor performance and microbial community analysis for the daily-fed reactors, in contrast to those fed bi-weekly. Changes in availability of glucose, and potentially other easily degradable sugars, due to natural fluctuations in the feedstock, could result in homolactic fermenters taking over. This hypothesis is strengthened by the fact that lactic acid peaks generally occurred at similar times in duplicate reactors which were subjected to the same feedstock variations (Figure 5-6).

5.1.4. Conclusion

This study has shown that feeding patterns in semi-continuous operation affected the acidogenic fermentation outcome and microbial community composition during food waste fermentation. A more stable product profile was obtained by operating sCSTR with a longer batch cycle time by feeding bi-weekly. A balance of lactic acid, ethanol and acetate fermentation was followed by chain elongation. By contrast, a daily feeding pattern resulted in less stable effluent composition with more fluctuation of lactic acid content, even though similar yields of overall liquid fermentation products were attained. Thus, operation at longer batch cycles promotes the formation of products resulting from chain elongation. This was coupled with reduced need for pH-correcting chemicals and hence reduction in operating and environmental costs.

Careful analysis of correlations in reactor performance and microbial community dynamics has shown that homolactic fermentation, and a heterolactic acetate-ethanol fermentation with chain elongation, are likely to be two competing pathways. From the cycle studies the former was more favoured by a daily feeding pattern, due to greater availability of easily biodegradable sugars. By contrast, a bi-weekly feeding pattern promoted higher yields of ethanol and n-caprylic acid. In order to maximize MCCA yields and process stability in acidogenic food waste fermentation, future work should work to improve understanding of the syntrophic and competitive interactions between different LAB and other fermentative bacteria. This is of particular importance when considering application of FW fermentation as a waste valorisation technology where feedstock variations and other small disturbances in operation are inevitable. In general, longer time in between feeding events in semi-continuous anaerobic fermentation of FW results in more stable MCCA product formation and reduced need for pH correction, and hence reduced operating costs.

Funding and acknowledgements

We would like to thank the support of and funding from Wessex Water and GENeco (Bristol, UK), and SCELSE (NTU, Singapore). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement H2020-MSCA-CO-FUND, No 665992. This work is supported by EPSRC under grant EP/L016354/1. The collaboration received additional travel funding granted from the Santander Postgraduate Mobility Award. The authors would like to thank Liew Chee Wai and the staff at Quad Café (NTU, Singapore) for reliable and regular support in the collection of samples, Eric Dubois Hill for helpful discussion regarding statistical analysis, and Eganathan Kaliyamoorthy and Sara Swa Thi for technical assistance in reactor operation and chemical analysis.

References

[1] X. Xiong *et al.*, "Value-added chemicals from food supply chain wastes: State-of-the-art review and future prospects," *Chemical Engineering Journal,* vol. 375, 2019.

[2] F. Cherubini, S. Bargigli, and S. Ulgiati, "Life cycle assessment (LCA) of waste management strategies: Landfilling, sorting plant and incineration," *Energy,* vol. 34, no. 12, pp. 2116-2123, 2009.

[3] X. Shi, G. Zheng, Z. Shao, and D. Gao, "Effect of source-classified and mixed collection from residential household waste bins on the emission characteristics of volatile organic compounds," *Sci Total Environ,* vol. 707, p. 135478, Mar 10 2020.

[4] G. Capson-Tojo, M. Rouez, M. Crest, J.-P. Steyer, J.-P. Delgenès, and R. Escudié, "Food waste valorization via anaerobic processes: a review," *Reviews in Environmental Science and Bio/Technology,* vol. 15, no. 3, pp. 499-547, 2016.

[5] M. Zhou, B. Yan, J. W. C. Wong, and Y. Zhang, "Enhanced volatile fatty acids production from anaerobic fermentation of food waste: A mini-review focusing on acidogenic metabolic pathways," *Bioresour Technol,* vol. 248, no. Pt A, pp. 68-78, Jan 2018.

[6] R. Moscoviz, E. Trably, N. Bernet, and H. Carrère, "The environmental biorefinery: state-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation," *Green Chemistry,* vol. 20, no. 14, pp. 3159-3179, 2018.

[7] M. G. Gänzle, "Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage," *Current Opinion in Food Science,* vol. 2, pp. 106-117, 2015.

[8] F. Asunis *et al.*, "Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey," *Bioresour Technol,* vol. 289, p. 121722, Oct 2019.

[9] M. T. Agler, B. A. Wrenn, S. H. Zinder, and L. T. Angenent, "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform," *Trends Biotechnol,* vol. 29, no. 2, pp. 70-8, Feb 2011.

[10] L. T. Angenent *et al.*, "Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals," *Environ Sci Technol,* vol. 50, no. 6, pp. 2796-810, Mar 15 2016.

[11] H. Fisgativa, A. Tremier, and P. Dabert, "Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion," *Waste Manag,* vol. 50, pp. 264-74, Apr 2016.

[12] C. O. Nzeteu, A. C. Trego, F. Abram, and V. O'Flaherty, "Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system," *Biotechnol Biofuels,* vol. 11, p. 108, 2018.

[13] C. A. Contreras-Davila, V. J. Carrion, V. R. Vonk, C. N. J. Buisman, and D. Strik, "Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation," *Water Res,* vol. 169, p. 115215, Feb 1 2020.

[14] V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting Organic Load as a Strategy to Direct Single-Stage Food Waste Fermentation from Anaerobic Digestion to Chain Elongation," *Processes,* vol. 8, no. 11, 2020.

[15] M. E. Hibbing, C. Fuqua, M. R. Parsek, and S. B. Peterson, "Bacterial competition: surviving and thriving in the microbial jungle," *Nat Rev Microbiol,* vol. 8, no. 1, pp. 15-25, Jan 2010.

[16] L. S. Serafim, P. C. Lemos, M. G. Albuquerque, and M. A. Reis, "Strategies for PHA production by mixed cultures and renewable waste materials," *Appl Microbiol Biotechnol,* vol. 81, no. 4, pp. 615-28, Dec 2008.

[17] X. Xu *et al.*, "Stabilizing lactate production through repeated batch fermentation of food waste and waste activated sludge," *Bioresour Technol,* vol. 300, p. 122709, Mar 2020.

[18] J. H. Park, G. Kumar, Y. M. Yun, J. C. Kwon, and S. H. Kim, "Effect of feeding mode and dilution on the performance and microbial community population in anaerobic digestion of food waste," *Bioresour Technol,* vol. 248, no. Pt A, pp. 134-140, Jan 2018.

[19] E. Nebot, L. I. Romero, J. M. Quiroga, and D. Sales, "Effect of feed frequency on the performance of anaerobic filters," *Anaerobe* vol. 1, pp. 113-120, 1995.

[20] A. M. Zealand, A. P. Roskilly, and D. W. Graham, "Effect of feeding frequency and organic loading rate on biomethane production in the anaerobic digestion of rice straw," *Applied Energy,* vol. 207, pp. 156-165, 2017.

[21] V. De Groof, M. Coma Bech, T. Arnot, D. Leak, and A. Lanham, "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters [submitted]," *Waste Management,* 2020.

[22] M. Coma *et al.*, "Organic waste as a sustainable feedstock for platform chemicals," *Faraday Discuss,* vol. 202, pp. 175-195, Sep 21 2017.

[23] G. Manni and F. Caron, "Calibration and determination of volatile fatty acids in waste leachates by gas chromatography," *Journal of Chromatography A,* vol. 690, pp. 237-242, 1995.

[24] APHA, "Standard methods for the examination of water and wastewater," vol. 23, 2017.

[25] M. Albertsen, S. M. Karst, A. S. Ziegler, R. H. Kirkegaard, and P. H. Nielsen, "Back to Basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities," *PLos ONE,* vol. 10, no. 7, p. e0132783, 2015.

[26] A. Apprill, S. McNally, R. Parsons, and L. Weber, "Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton," *Aquatic Microbial Ecology,* vol. 75, no. 2, pp. 129-137, 2015.

[27] B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. Johnson, and S. P. Holmes, "DADA2: High-resolution sample inference from Illumina amplicon data," *Nat Methods,* vol. 13, no. 7, pp. 581-3, Jul 2016.

[28] C. Quast *et al.*, "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools," *Nucleic Acids Res,* vol. 41, no. Database issue, pp. D590-6, Jan 2013.

[29] R Core Team, "R: A language and envionment for statistical computing," ed. Vienna, Austria: R Foundation for Statistical Computing, 2017.

[30] P. Dalgaard, *Introductory Statistics with R*, 2 ed. New York: Springer Science+Business Media, 2008.

[31] Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," *Journal of the Royal Statistical Society. Series B (Methodological),* vol. 57, no. 1, pp. 289-300, 1995.

[32] H. Wickham, *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag, 2016.

[33] K. S. Andersen, R. H. Kirkegaard, S. M. Karst, and M. Albertsen, "Ampvis2: an R package to analyse and visualise 16S rRNA amplicon data," *bioRxiv,* 2018.

[34] J. Oksanen *et al.*, "Package 'vegan': Community Ecology Package (version 2.5-6)," ed, 2019.

[35] S. Weiss *et al.*, "Normalization and microbial differential abundance strategies depend upon data characteristics," *Microbiome,* vol. 5, no. 1, p. 27, Mar 3 2017.

[36] R. Lucas, J. Groeneveld, H. Harms, K. Johst, K. Frank, and S. Kleinsteuber, "A critical evaluation of ecological indices for the comparative analysis of microbial communities based on molecular datasets," *FEMS Microbiol Ecol,* vol. 93, no. 1, Jan 2017.

[37] S. H. Hurlbert, "The nonconcept of species diversity: a critique and alternative parameters," *Ecology,* vol. 52, pp. 577-586, 1971.

[38] L. Jost, "Entropy and diversity," *Oikos,* vol. 113, no. 2, pp. 363-375, 2006.

[39] E. Santillan, H. Seshan, F. Constancias, D. I. Drautz-Moses, and S. Wuertz, "Frequency of disturbance alters diversity, function, and underlying assembly mechanisms of complex bacterial communities," *NPJ Biofilms Microbiomes,* vol. 5, no. 1, p. 8, 2019.

[40] M. De Caceres and P. Legendre, "Associations between species and groups of sites: indices and statistical interference," *Ecology,* vol. 90, no. 12, pp. 3566-3574, 2010.

[41] P. L. Buttigieg and A. Ramette, "A guide to statistical analysis in microbial ecology: a community-focused, living review of multivariate data analyses," *FEMS Microbiol Ecol,* vol. 90, no. 3, pp. 543-50, Dec 2014.

[42] J. Quensen, "Package 'ggordiplots': make ggplot Versions of Vegan's Ordiplots," ed, 2020.

[43] M. J. Anderson and D. C. I. Walsh, "PERMANOVA, ANOSIM and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing?," *Ecological Monographs,* vol. 83, no. 4, pp. 557-574, 2013.

[44] M. J. Anderson, "Distance-based tests for homogeneity of multivariate dispersions," *Biometrics,* vol. 62, no. 1, pp. 245-53, Mar 2006.

[45] F. E. Harrell Jr and C. Dupont, "Package 'Hmisc' version 4.4-1," ed: CRAN, 2020.

[46] T. Wei and V. Simko, "R package "corplot": Visualization of a Correlation Matrix (version 0.84)," ed, 2017.

[47] C. M. Spirito, A. M. Marzilli, and L. T. Angenent, "Higher Substrate Ratios of Ethanol to Acetate Steered Chain Elongation toward n-Caprylate in a Bioreactor with Product Extraction," *Environ Sci Technol,* vol. 52, no. 22, pp. 13438-13447, Nov 20 2018.

[48] J. M. Carvajal-Arroyo, S. J. Andersen, R. Ganigué, R. A. Rozendal, L. T. Angenent, and K. Rabaey, "Production and extraction of medium chain carboxylic acids at a semi-pilot scale," *Chemical Engineering Journal,* 2020.

[49] M. Coma, R. Vilchez-Vargas, H. Roume, R. Jauregui, D. H. Pieper, and K. Rabaey, "Product Diversity Linked to Substrate Usage in Chain Elongation by Mixed-Culture Fermentation," *Environ Sci Technol,* vol. 50, no. 12, pp. 6467-76, Jun 21 2016.

[50] L. A. Kucek, M. Nguyen, and L. T. Angenent, "Conversion of L-lactate into n-caproate by a continuously fed reactor microbiome," *Water Res,* vol. 93, pp. 163-171, Apr 15 2016.

[51] P. Candry, L. Radic, J. Favere, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Mildly acidic pH selects for chain elongation to caproic acid over alternative pathways during lactic acid fermentation," *Water Res,* vol. 186, p. 116396, Sep 7 2020.

[52] J. Lambrecht *et al.*, "Key sub-community dynamics of medium-chain carboxylate production," *Microb Cell Fact,* vol. 18, no. 1, p. 92, May 28 2019.

[53] J. Tang, X. Wang, Y. Hu, Y. Zhang, and Y. Li, "Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR," *Waste Manag,* vol. 52, pp. 278-85, Jun 2016.

[54] Y. Itoh, K. Tada, T. Kanno, and J. Horiuchi, "Selective production of lactic acid in continuous anaerobic acidogenesis by extremely low pH operation," *J Biosci Bioeng,* vol. 114, no. 5, pp. 537-9, Nov 2012.

[55] W. S. Chen, D. Strik, C. J. N. Buisman, and C. Kroeze, "Production of Caproic Acid from Mixed Organic Waste: An Environmental Life Cycle Perspective," *Environ Sci Technol,* vol. 51, no. 12, pp. 7159-7168, Jun 20 2017.

[56] C. Sundberg *et al.*, "454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters," *FEMS Microbiol Ecol,* vol. 85, no. 3, pp. 612-26, Sep 2013.

[57] J. De Vrieze *et al.*, "The full-scale anaerobic digestion microbiome is represented by specific marker populations," *Water Res,* vol. 104, pp. 101-110, Nov 1 2016.

[58] M. Gantar and Z. Svircev, "Microalgae and Cyanobacteria: Food for Thought," *J Phycol,* vol. 44, no. 2, pp. 260-8, Apr 2008.

[59] C. Sundberg *et al.*, "Characterisation of source-separated household waste intended for composting," *Bioresour Technol,* vol. 102, no. 3, pp. 2859-67, Feb 2011.

[60] V. Fusco *et al.*, "The genus Weissella: taxonomy, ecology and biotechnological potential," *Front Microbiol,* vol. 6, p. 155, 2015.

[61] G. Novik, O. Meerovskaya, and V. Savich, "Waste degradation and utilization by lactic acid bacteria: Use of lactic acid bacteria in production of food additives, bioenergy and biogas," in *Food additives*, D. N. Karunaratne and G. Pamunuwa, Eds. : IntechOpen, 2017.

[62] S. E. Daly, J. G. Usack, L. A. Harroff, J. G. Booth, M. P. Keleman, and L. T. Angenent, "Systematic Analysis of Factors That Affect Food-Waste Storage: Toward Maximizing Lactate Accumulation for Resource Recovery," *ACS Sustainable Chemistry & Engineering,* vol. 8, no. 37, pp. 13934-13944, 2020.

[63] S. Im, M.-K. Lee, Y.-M. Yun, S.-K. Cho, and D.-H. Kim, "Effect of storage time and temperature on hydrogen fermentation of food waste," *International Journal of Hydrogen Energy,* vol. 45, no. 6, pp. 3769-3775, 2020.

[64] F. Lu, X. Xu, L. Shao, and P. He, "Importance of storage time in mesophilic anaerobic digestion of food waste," *J Environ Sci (China),* vol. 45, pp. 76-83, Jul 2016.

[65] A. Duber, R. Zagrodnik, J. Chwialkowska, W. Juzwa, and P. Oleskowicz-Popiel, "Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation," *Sci Total Environ,* vol. 728, p. 138814, Aug 1 2020.

[66] J. De Vrieze *et al.*, "Stochasticity in microbiology: managing unpredictability to reach the Sustainable Development Goals," *Microb Biotechnol,* vol. 13, no. 4, pp. 829-843, Jul 2020.

[67] J. Zheng *et al.*, "A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae," *Int J Syst Evol Microbiol,* Apr 15 2020.

[68] M. J. Scarborough, K. S. Myers, T. J. Donohue, and D. R. Noguera, "Medium-Chain Fatty Acid Synthesis by "Candidatus Weimeria bifida" gen. nov., sp. nov., and "Candidatus Pseudoramibacter fermentans" sp. nov," *Appl Environ Microbiol,* vol. 86, no. 3, Jan 21 2020.

[69] B. Liu, S. Kleinsteuber, F. Centler, H. Harms, and H. Strauber, "Competition Between Butyrate Fermenters and Chain-Elongating Bacteria Limits the Efficiency of Medium-Chain Carboxylate Production," *Front Microbiol,* vol. 11, p. 336, 2020.

[70] M. J. Scarborough, G. Lynch, M. Dickson, M. McGee, T. J. Donohue, and D. R. Noguera, "Increasing the economic value of lignocellulosic stillage through medium-chain fatty acid production," *Biotechnol Biofuels,* vol. 11, p. 200, 2018.

[71] H. Uematsu and E. Hoshino, "Degradation of Arginine and Other Amino Acids byEubacterium nodatumATCC 33099," *Microbial Ecology in Health and Disease,* vol. 9, no. 6, pp. 305-311, 2009.

[72] X. Y. Gu, J. Z. Liu, and J. W. C. Wong, "Control of lactic acid production during hydrolysis and acidogenesis of food waste," *Bioresour Technol,* vol. 247, pp. 711-715, Jan 2018.

[73] L. R. Jarboe, L. A. Royce, and P. Liu, "Understanding biocatalyst inhibition by carboxylic acids," *Front Microbiol,* vol. 4, p. 272, Sep 3 2013.

[74] M. Roghair *et al.*, "Effect of n-Caproate Concentration on Chain Elongation and Competing Processes," *ACS Sustain Chem Eng,* vol. 6, no. 6, pp. 7499-7506, Jun 4 2018.

[75] J. L. Rombouts, E. M. M. Kranendonk, A. Regueira, D. G. Weissbrodt, R. Kleerebezem, and M. C. M. van Loosdrecht, "Selecting for lactic acid producing and utilising bacteria in anaerobic enrichment cultures," *Biotechnol Bioeng,* vol. 117, no. 5, pp. 1281-1293, May 2020.

[76] J.-H. Park, D.-H. Kim, S.-H. Kim, J.-J. Yoon, and H.-D. Park, "Effect of substrate concentration on the competition between Clostridium and Lactobacillus during biohydrogen production," *International Journal of Hydrogen Energy,* vol. 43, no. 25, pp. 11460-11469, 2018.

[77] A. Goel *et al.*, "Protein costs do not explain evolution of metabolic strategies and regulation of ribosomal content: does protein investment explain an anaerobic bacterial Crabtree effect?," *Mol Microbiol,* vol. 97, no. 1, pp. 77-92, Jul 2015.

[78] H. Bachmann, D. Molenaar, F. Branco Dos Santos, and B. Teusink, "Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria," *FEMS Microbiol Rev,* vol. 41, no. Supp_1, pp. S201-S219, Aug 1 2017.

5.2. Supplementary information

Feeding pattern affects balance between lactic acid fermentation and microbial chain elongation

5.2.1. Feedstock characterisation and storage

Table S5-1 *Summary of COD and solids content of the feedstock used throughout continuous operation. Averages (AVG), standard deviations (SD), relative SD (RSD) and minima (Min) and maxima (Max) were determined over 37 mixtures of feed that were prepared throughout reactor operation comprising a blend of cafeteria food waste (FW) and soybean soaking wastewater (Soy).*

	Feed day 0-69				Feed day 70-122			
	AVG ± SD	**RSD**	**Min**	**Max**	**AVG ± SD**	**RSD**	**Min**	**Max**
FW/Soy (kg L^{-1})	0.5				0.38			
tCOD (gCOD L^{-1})	132 ± 19	14%	97	167	90 ± 19	22%	62	118
sCOD (gCOD L^{-1})	49 ± 9	19%	34	68	45 ± 8	18%	33	65
TS (g L^{-1})	100 ± 8	8%	91	121	82 ± 6	7%	86	118
VS (g L^{-1})	97 ± 9	9%	76	93	78 ± 5	7%	71	89

Table S5-2 *To determine the effect of substrate storage a blend of 0.5 kg FW and 1 L Soy (FW:Soy), and pure Soy were characterised when freshly prepared, and after 3, 7 or 8 days of storage at 4 °C. Values represent averages of analytical duplicates and their standard deviation.*

		FW:Soy			Soy	
	unit	**Fresh**	**3 days**	**7 days**	**Fresh**	**8 days**
pH	--	6.24 ± 0.02	4.49 ± 0.02	4.20 ± 0.01	6.01 ± 0.02	5.91 ± 0.01
TS (*TSS)	g L^{-1}	93.3 ± 0.7	95.4 ± 1.1	93.6 ± 0.8	*0.056 ± 0.000	*0.050 ± 0.006
VS (*VSS)	g L^{-1}	89.7 ± 0.6	91.8 ± 1.1	90.0 ± 0.7	*0.043 ± 0.001	*0.041 ± 0.012
VS/TS (*VSS/TSS)	%	96 ± 1	96 ± 0	96 ± 0	*77 ± 3	*80 ± 15
Total COD (tCOD)	gCOD L^{-1}	127 ± 8	120 ± 22	na ± na	1.39 ± 0.06	1.42 ± 0.02
Soluble COD (sCOD)	gCOD L^{-1}	43.5 ± 0.4	55.2 ± 0.3	na ± na	1.51 ± 0.01	1.46 ± 0.00
Glucose	g L^{-1}	1.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.00	0.18 ± 0.00
Sugars**	g L^{-1}	0.77 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.00	0.09 ± 0.00
Lactic acid	g L^{-1}	0.19 ± 0.01	1.84 ± 0.02	2.78 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
Ethanol	g L^{-1}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.00	0.16 ± 0.00
Carboxylic acids	g L^{-1}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

**Calibrated using fructose, incl. sucrose and xylose

Within three days, the easily degradable sugars present in FW:Soy are fermented to lactic acid leading to acidification of the feedstock during storage. Soy without FW acidified to a much lesser extent, even after 8 days of storage at 4 °C. Solid and COD content did not change significantly during storage, suggesting hydrolysis in Soy was limited.

5.2.2. Reactor operation

Table S5-3 *Summary of operating conditions for the four semi-continuous stirred tank reactors (sCSTR) during the different operational phases. The average hydraulic retention time (HRT) was maintained throughout with minor variations resulting from variations in pumping volume. From Day 0 to 84, two sCSTR were being fed twice a week ($sCSTR_{BW}$) and two sCSTR were fed daily ($sCSTR_{D}$). From day 84 onwards, i.e., after 8 HRT, the $sCSTR_{D}$ were changed to twice a week feeding ($sCSTR_{BW\text{-}new}$) similar to $sCSTR_{BW}$. Feedstock varied as outlined in Table S5-1. On Day 70 onwards the COD and solids in the feedstock were reduced to prevent lactic acid accumulation, thus lowering average organic loading rate (OLR), and instantaneous organic load (IOL).*

Parameter ***Time of operation***	**$sCSTR_{BW}$ 1**	**$sCSTR_{BW}$ 2**	**$sCSTR_{D/BW\text{-}new}$ 1**	**$sCSTR_{D/BW\text{-}new}$ 2**
HRT (days)	10.3 ± 0.6	10.3 ± 0.6	10.9 ± 1.2	10.8 ± 1.1
Feeding strategy				
Day 0 - 83	Bi-weekly	Bi-weekly	Daily	Daily
Day 84-end	Bi-weekly	Bi-weekly	Bi-weekly	Bi-weekly
Fermentation cycle (days)				
Day 0 - 83	3.5 ± 0.2	3.5 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
Day 84-end	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.2
OLR (gCOD L^{-1} d^{-1})				
Day 0 - 69	13 ± 2	13 ± 2	12 ± 2	12 ± 2
Day 70-end	9 ± 2	9 ± 2	9 ± 2	9 ± 2
IOL (gCOD L^{-1})				
Day 0 - 69	46 ± 6	46 ± 6	12± 2	12 ± 2
Day 70-83	37 ± 3	37 ± 3	10.3 ± 0.5	9.7 ± 0.4
Day 84-end	29 ± 5	29 ± 5	29 ± 5	29 ± 5

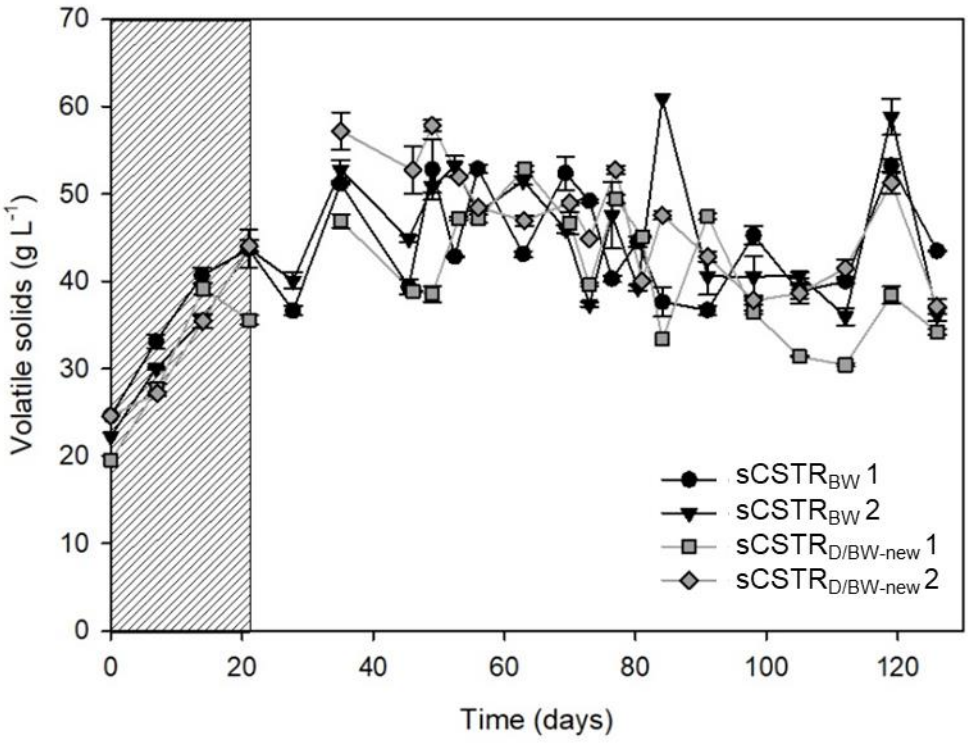


Figure S5-1 *Volatile solids (VS) concentration of effluent from duplicate semi-continuous stirred tank reactors fed bi-weekly ($sCSTR_{BW}$) or fed daily for the first 84 days of operation before switching to bi-weekly feeding ($sCSTR_{D/BW\text{-}new}$). Marked area was selected as the start-up phase after which VS varied less than 16% for all reactors.*

5.2.3. Microbial community analysis

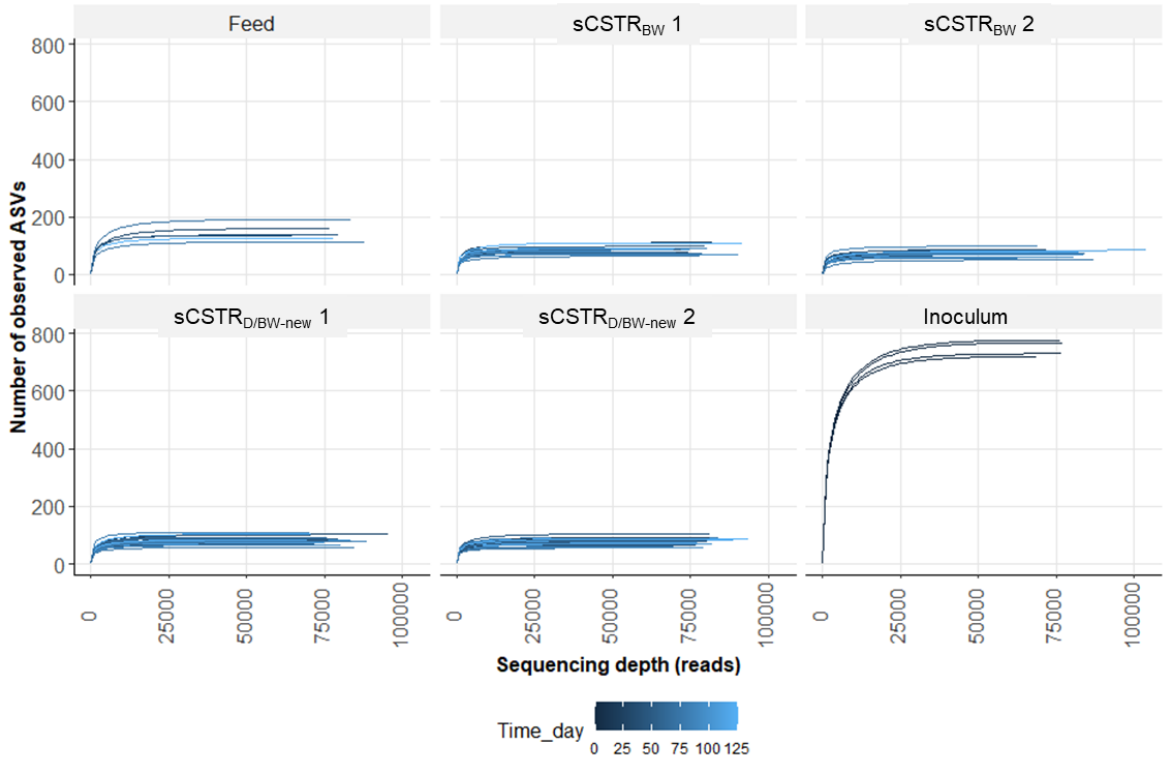


Figure S5-2 *Rarefaction curves calculated as the number of observed ASVs as function of the sequencing depth. Curves are grouped per samples taken from the feedstock (Feed), the reactors (sCSTR$_{BW}$ 1, 2 and sCSTR$_{D/BW}$ 1 and 2) and at the start of reactor operation after inoculation (inoculum). For further analysis, data was rarefied to 65,749 reads, which was the minimum sequencing depth obtained for one of the samples and located within the flattening range for all curves. Flattening of the curve indicates exhaustive sequencing of the diversity in the samples.*

5.2.4. General reactor performance

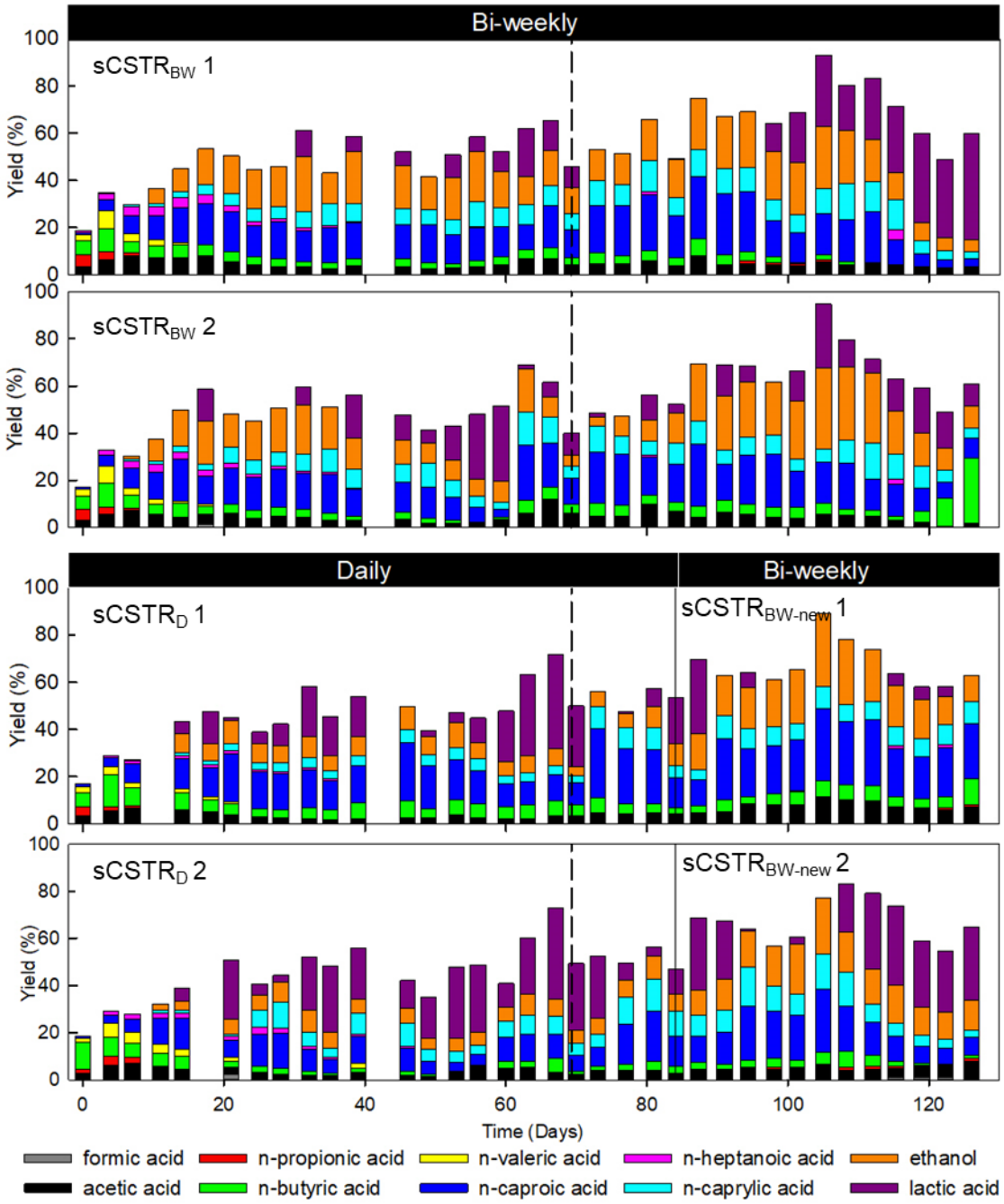


Figure S5-3 *Product yields from co-fermentation of food waste and soybean soaking wastewater in four sCSTR fed twice a week, or daily as indicated on top of the graphs. The dashed lines represent the day from which OLR was reduced from 13 to 9 ± 2 gCOD $L^{-1}d^{-1}$ to prevent lactic acid accumulation and improve overall conversion.*

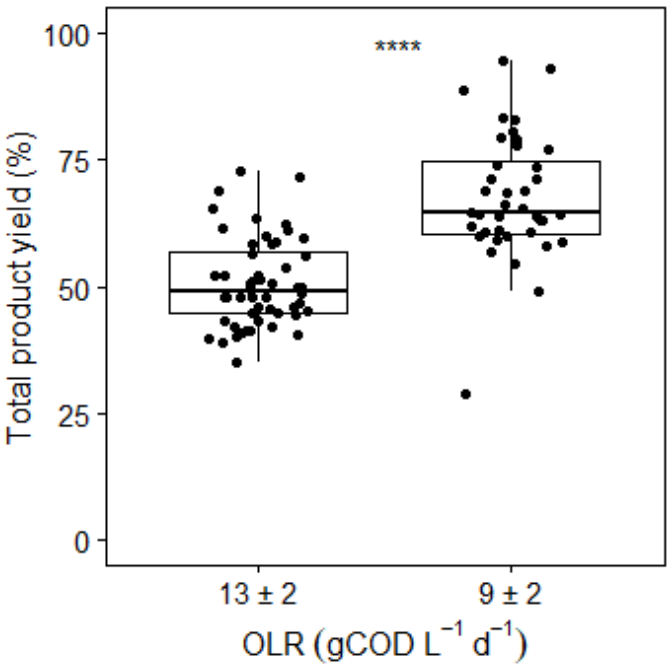


Figure S5-4 *Total product yield during operation of the four reactors at an organic loading rate (OLR) of 13 ± 2 gCOD $L^{-1}d^{-1}$ (number of samples, n=52) and 9 ± 2 gCOD $L^{-1}d^{-1}$ (n=40). Data from reactor start-up is excluded. Symbol "****" represents p-values of < 0.0001, resulting from a Wilcoxon-Mann-Whitney test corrected for a FDR of 5%.*

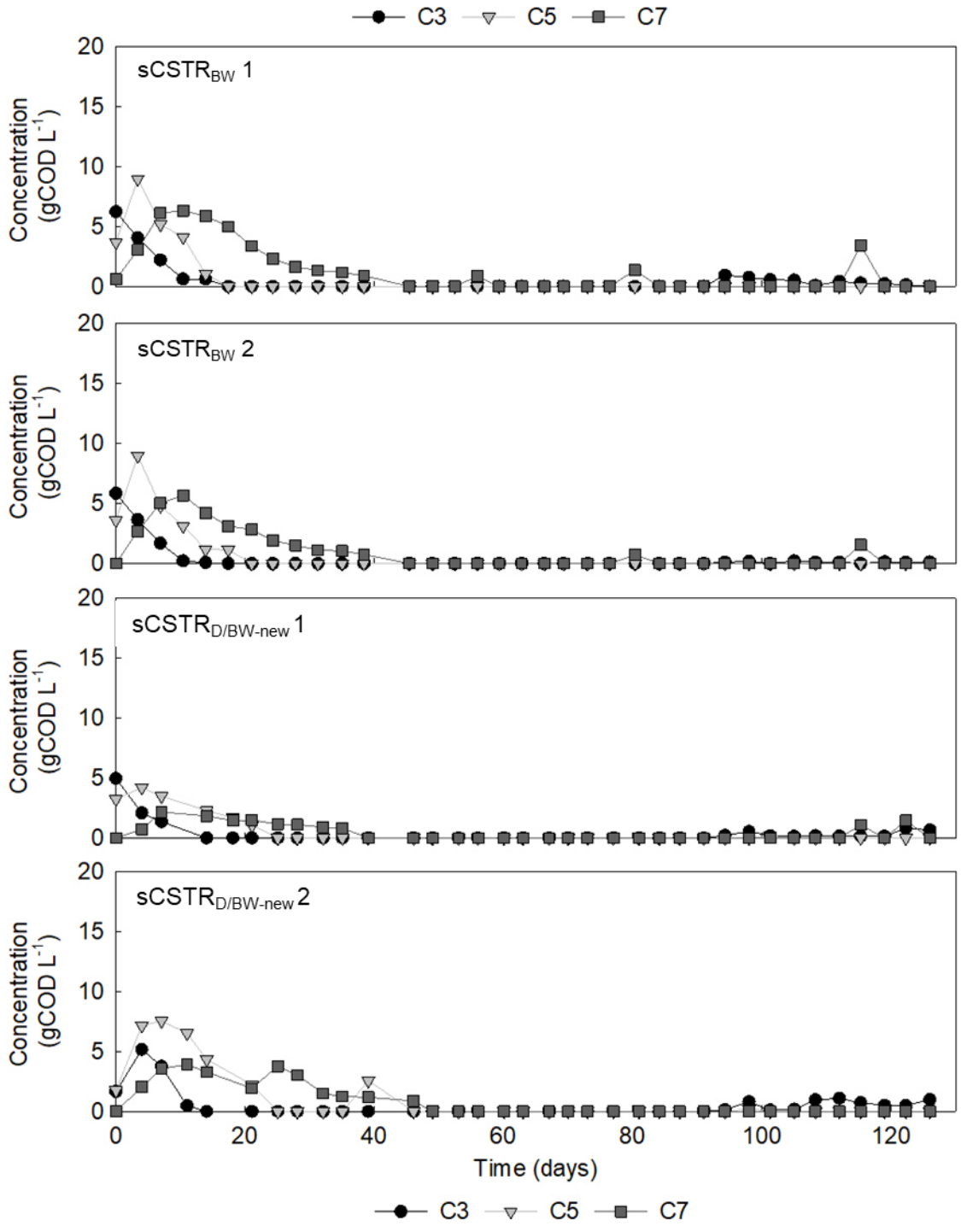


Figure S5-5 *Concentration profiles of the uneven carboxylic acids in the effluent of the semi-continuous reactors fed bi-weekly (sCSTR$_{BW}$) or fed daily for the first 8 HRT of operation (84 days) before switching to bi-weekly feeding (sCSTR$_{D/BW\text{-}new}$).*

5.2.5. Statistical analysis on reactor performance

5.2.5.1. Comparing product concentration per feeding pattern

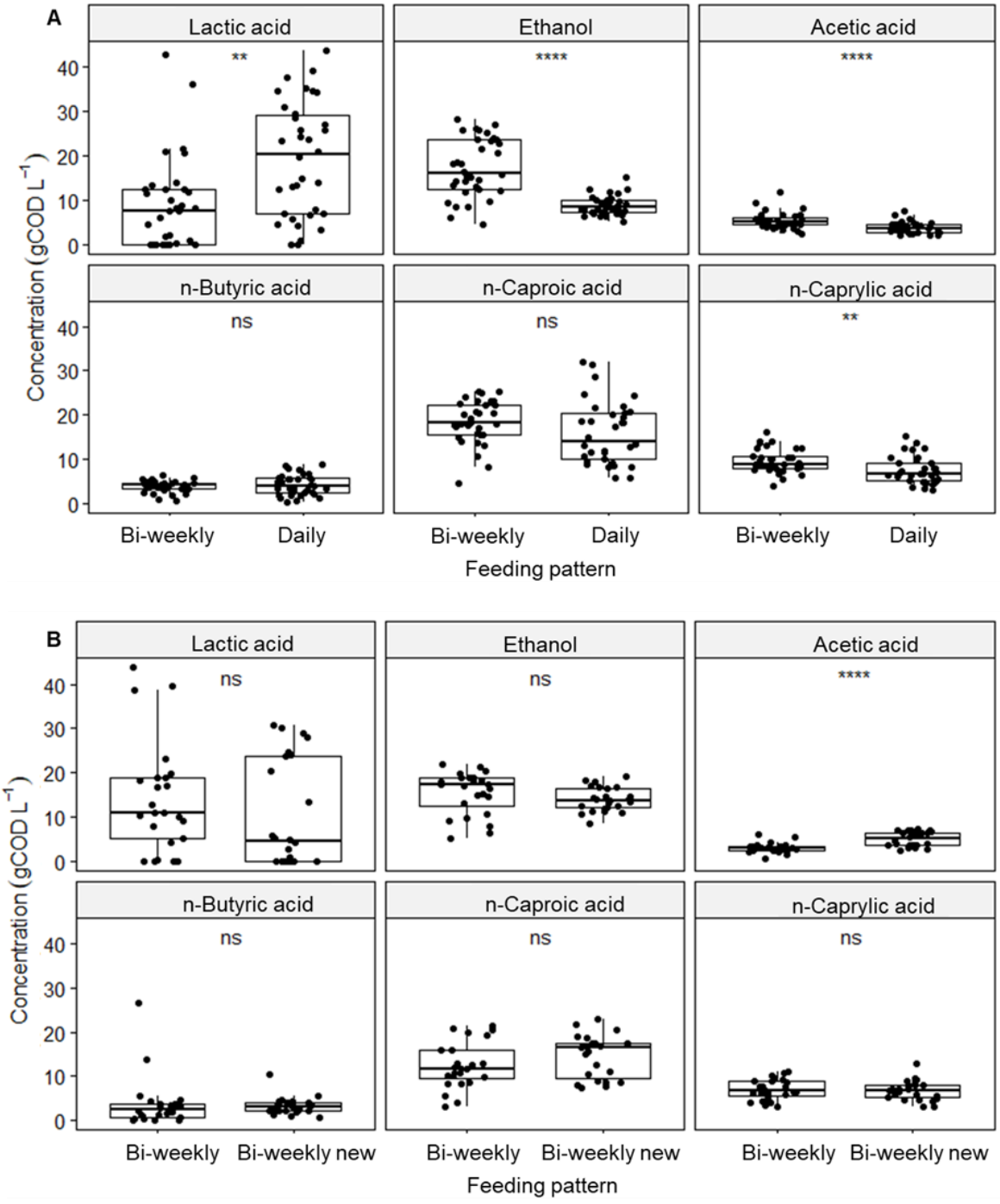


Figure S5-6 *Evaluation of product concentrations in the effluent per feeding pattern.* ***A:*** *bi-weekly and daily feeding is compared during the first 8 HRT of operation (excluding start-up).* ***B:*** *yields from after 8 HRT to end of operation are compared between reactors fed bi-weekly and reactors that were previously fed daily and switched to bi-weekly feeding (Bi-weekly new). Symbols "ns", "**", "***" and "****" represent p-values of >0.5 (not significant), <0.01, <0.001 and <0.0001, respectively, resulting from a Wilcoxon-Mann-Whitney test and corrected for multiple comparisons with a FDR of 5%.*

5.2.5.2. Comparing performance of duplicates

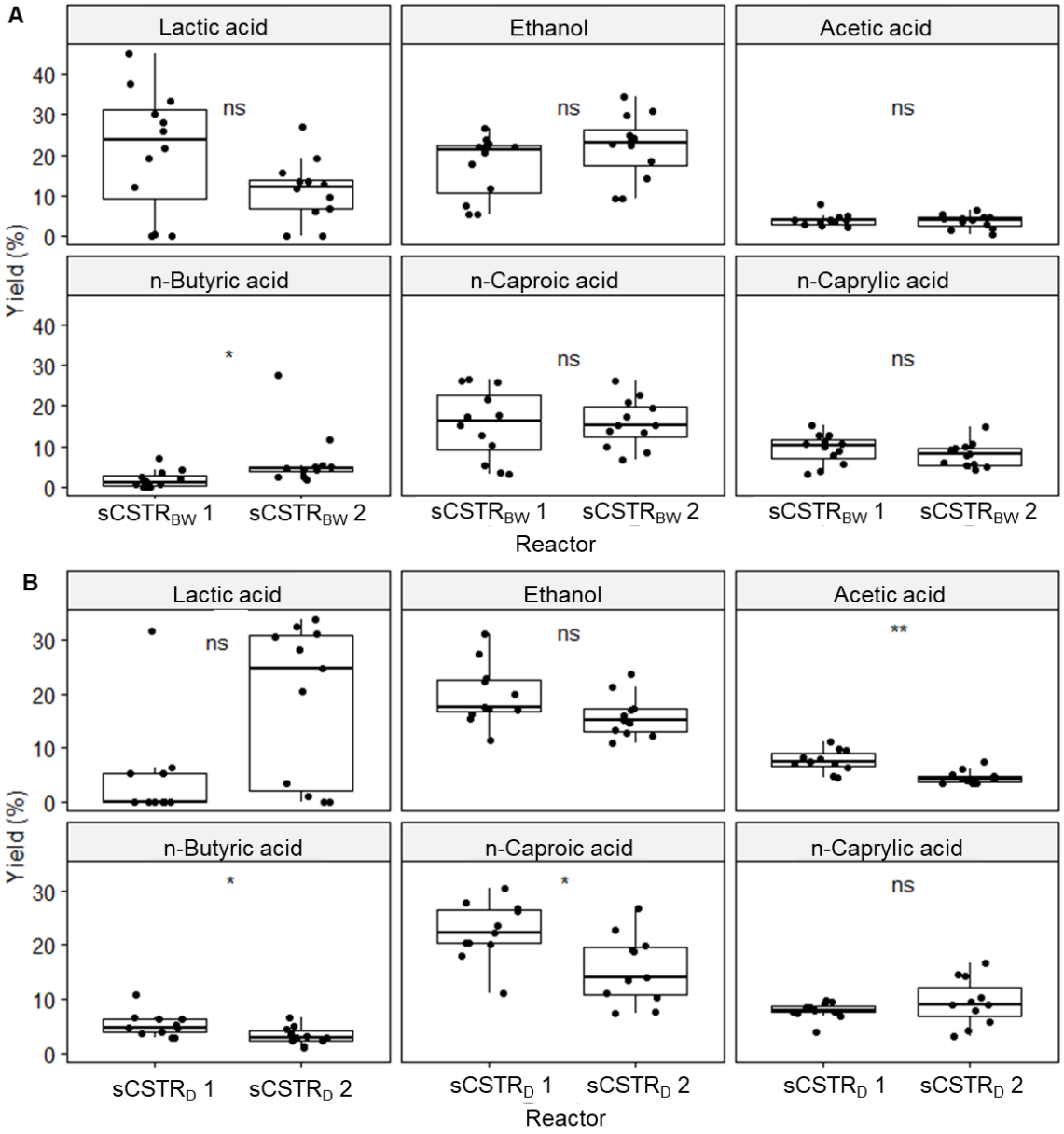


Figure S5-7 *Performances of duplicate reactors from after 8 HRT of operation until end were compared.* ***A****: the duplicate reactors that were fed bi-weekly (sCSTR$_{BW}$ 1 and 2).* ***B****: the duplicate reactors that were previously fed daily and had switched to a bi-weekly feeding pattern (sCSTR$_{BW\text{-}new}$ 1 and 2). Data Symbols "ns", "**" and "***" represent p-values of > 0.5, < 0.01 and < 0.001, respectively, resulting from a Wilcoxon-Mann-Whitney test and corrected for multiple comparisons with a FDR of 5%.*

5.2.5.3. Correlation analysis

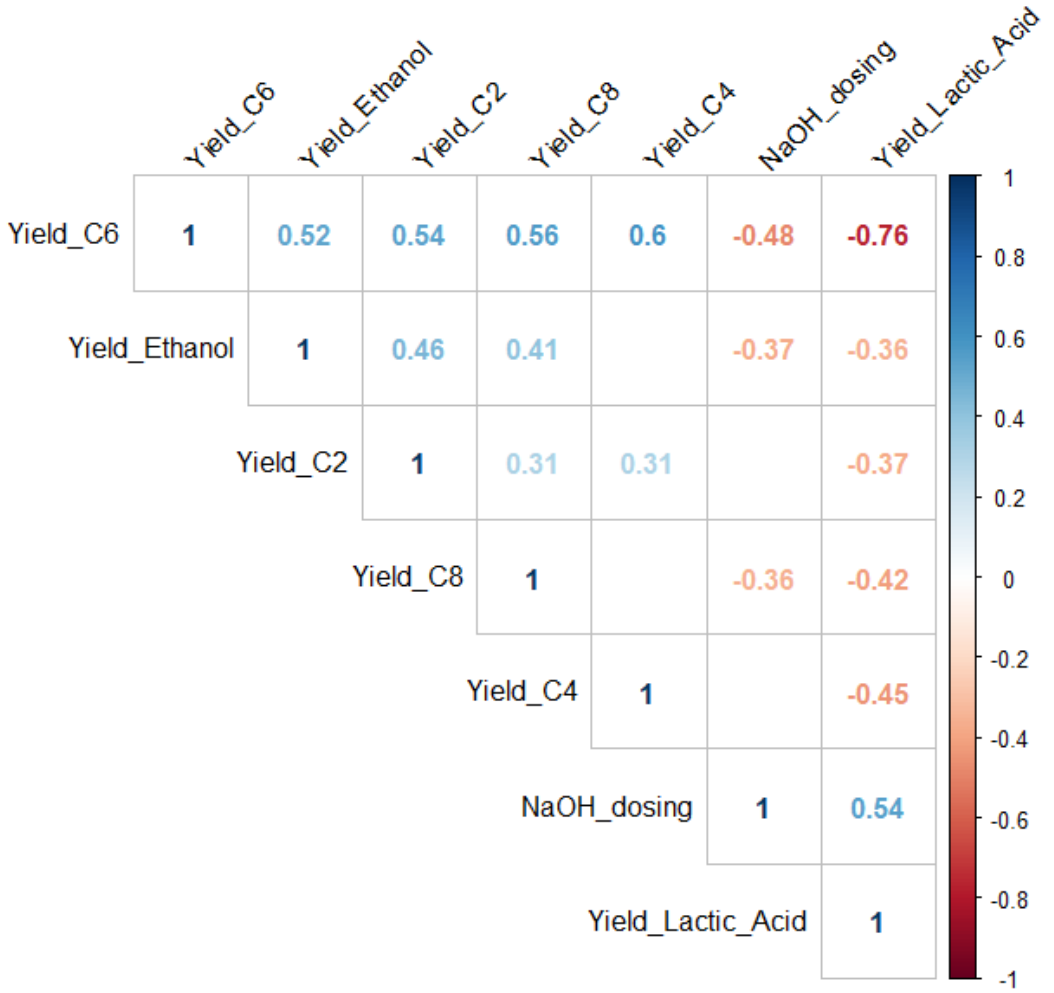


Figure S5-8 *Spearman correlation matrix of product yields and acidification in all four reactors. Variables on the left of the graph are significantly correlated (p<0.01) with the variables on the top by the correlation coefficient (r_S) given in the overlapping squares. The level of acidification during fermentation is presented by the amount of NaOH required to maintain minimum pH (NaOH dosing in g $L^{-1}d^{-1}$). The closer the r_S is to 1 (blue) or -1 (red), the more the two variables are positively or negatively correlated. C2: acetic acid; C4: n-butyric acid; C6: n-caproic acid; C8: n-caprylic acid. The p-values from all correlation analysis were corrected for multiple comparisons using a FDR of 5%.*

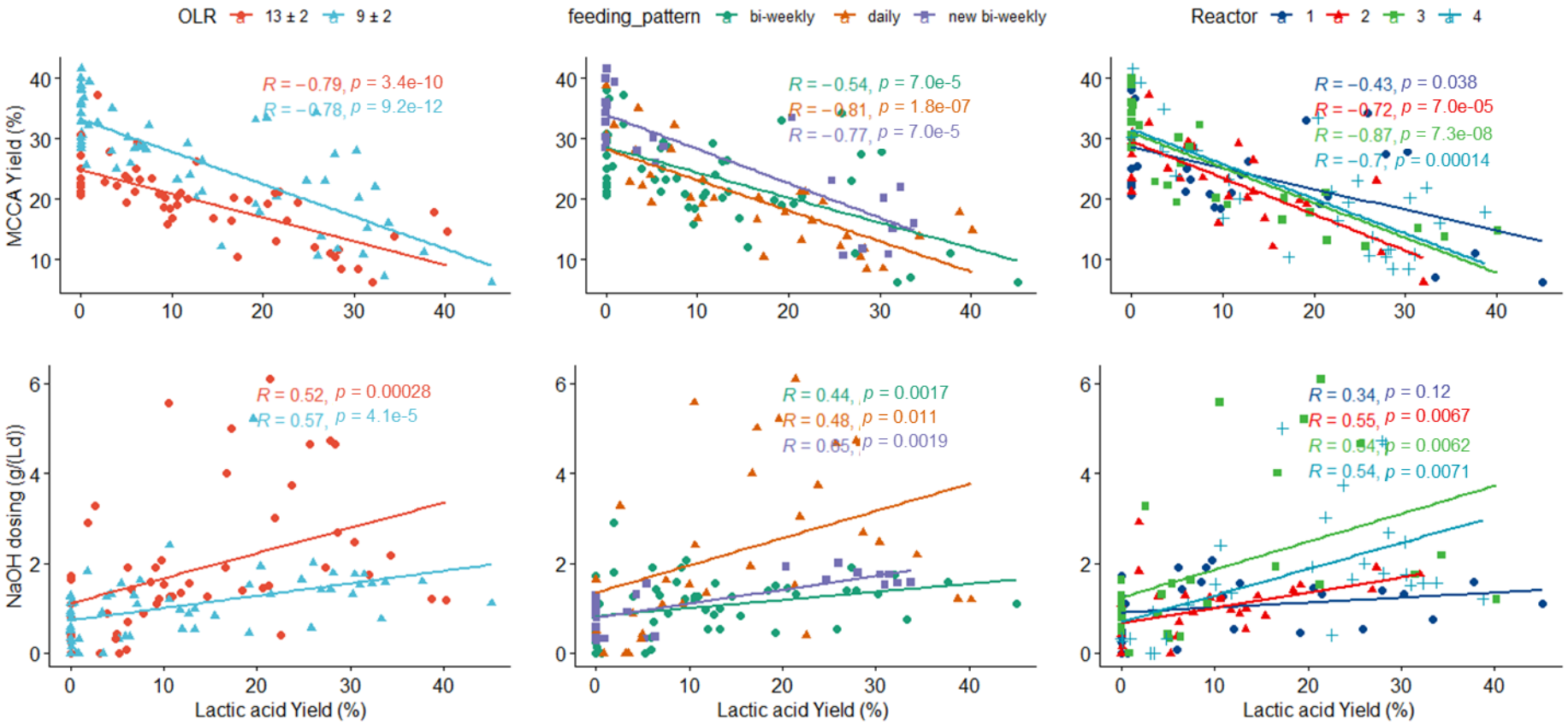


Figure S5-9 *Lactic acid yields correlated negatively with MCCA yield (top) and positively with NaOH dosing (bottom). Spearman correlation coefficient (R) and significance of correlation (p-value) is determined on the variables per organic loading rate (OLR, left), per type of feeding pattern (middle) and per reactor (right). The p-values from all correlation analysis were corrected for multiple comparisons using a FDR of 5%.*

5.2.6. Additional statistical data on microbial community analysis

Table S5-4 *Evaluating significant difference between microbial community structure according to feeding pattern. Multivariate tests (PERMANOVA and PERMDISP) were performed on Hellinger transformed relative abundances of communities using feeding pattern as factor and Bray-Curtis dissimilarities. The p-values were calculated using 9,999 permutations and corrected for multiple comparisons using a FDR of 5%. Bi-weekly and daily fed reactors are compared for sample points during the first 8 HRT of operation excluding start-up, and bi-weekly fed reactors are compared with reactors switched from daily feeding to bi-weekly (Bi-weekly new) in samples taken after 8 HRT of operation. PERMDISP p-values >0.05 ensure assumption of homogeneity of multivariate dispersion is met.*

		PERMANOVA			PERMDISP	
Comparison	**n**	**Df**	**Pseudo-F**	**p**	**Df**	**F**	**p**
Bi-weekly VS. Daily	36	1	8.8656	0.0004	1	0.0034	0.96
Bi-weekly VS. Bi-weekly new	28	1	2.8974	0.0024	1	0.0217	0.96

n, number of samples; Df, degree of freedom

Table S5-5 *Indicator species analysis on ASVs with a relative abundance >1% per feeding pattern. Comparison 1 determines the indicator species for two reactors that were fed bi-weekly compared to two reactors that were fed daily during the first 8 HRT of operation (excluding start-up). Comparison 2 determines the indicator species for two reactors that were fed bi-weekly compared to two reactors that were previously fed daily but switched to bi-weekly feeding (Bi-weekly new) after 8 HRT of operation until end. The p-values were calculated using 9,999 permutations and corrected for multiple comparisons using a FDR of 5%.*

	n	**Genus**	**Stat**	**p-value**
Comparison 1				
Bi-weekly	18			
ASV_8		*Olsenella*	0.615	0.001
ASV_6		*Pseudoramibacter*	0.545	0.002
ASV_7		*[Eubacterium] nodatum group*	0.491	0.002
Daily	18			
ASV_2		*Lactobacillus*	0.582	0.002
ASV_4		*Olsenella*	0.508	0.005
Comparison 2				
Bi-weekly	14			
ASV_13		*Acetitomaculum*	0.441	0.02
Bi-weekly new	14			
ASV_14		*Olsenella*	0.519	0.002

n, number of samples; Stat, strength of association

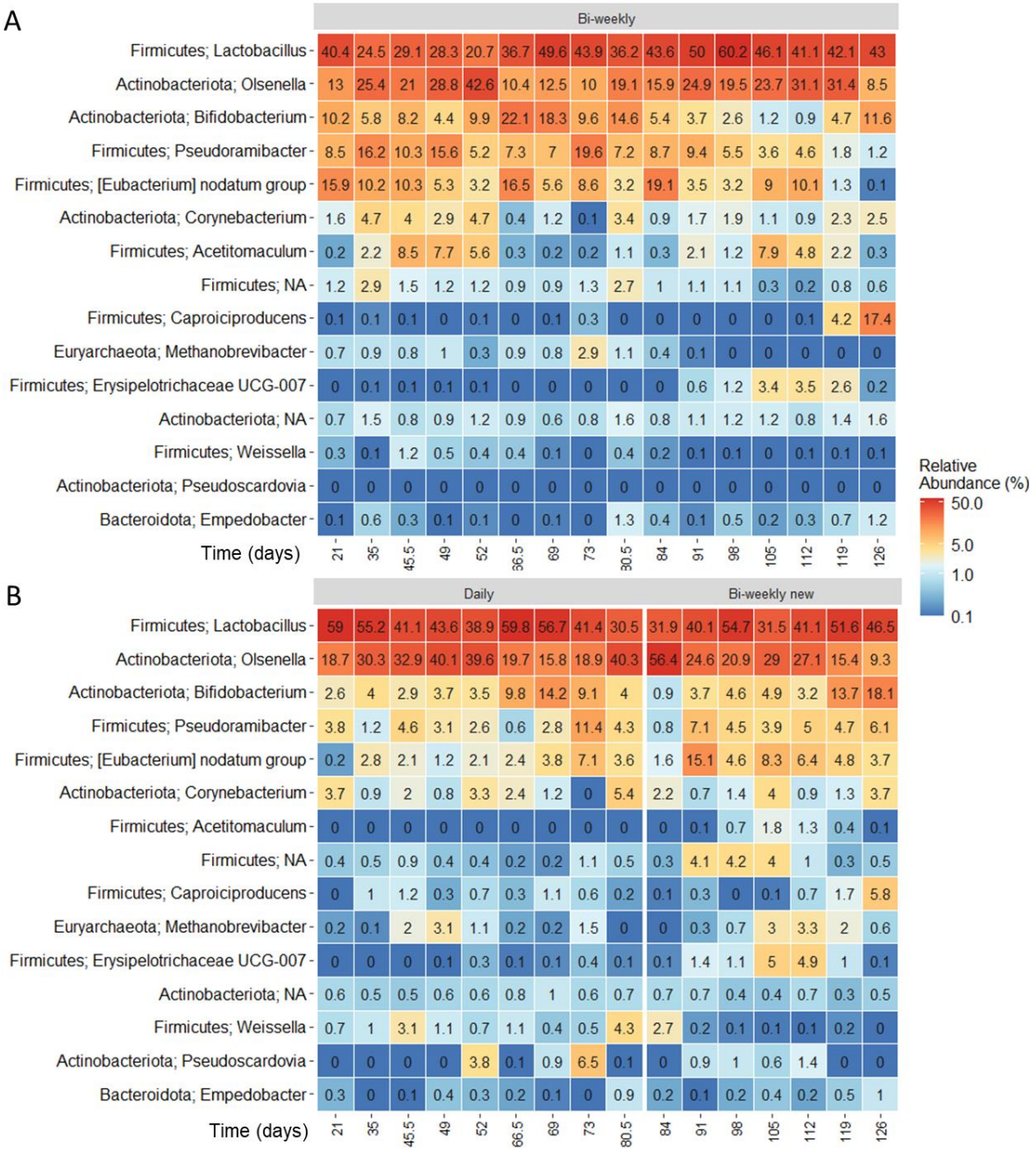


Figure S5-10 *Heatmap of the relative abundance of the genera in the MMC of the different samples over time in (**A**) the bi-weekly fed reactors and (**B**) the reactors fed daily and switched to bi-weekly feeding after 8 HRT as indicated on top of the graph. Only genera with an average relative abundance >1% are shown.*

Table S5-6 *Overview of the correlation analysis between the most abundant ASVs in the reactors with reactor performance, calculated over 64 samples. The spearman correlation coefficient (r_S) and the p-value of correlation are presented for each ASV in respect with its correlation to the yield of lactic acid (LA), ethanol (EtOH), acetic acid (C2), n-butyric acid (C4), n-caproic acid (C6) or n-caprylic acid (C8). Colour intensity highlights strength where red indicates a negative and green a positive correlation. The p-values were corrected for multiple comparisons using a FDR of 5%.*

		LA	EtOH	C2	C4	C6	C8
Lactobacillus; ASV 1	p	< 0.01	< 0.0001	< 0.0001	ns	< 0.01	ns
	r_S	-0.36	0.72	0.49		0.41	
Lactobacillus; ASV 2	p	< 0.0001	< 0.0001	< 0.01	> 0.05	< 0.0001	< 0.001
	r_S	0.52	-0.77	-0.40		-0.47	-0.44
Olsenella; ASV 3	p	<0.05	ns	ns	ns	ns	ns
	r_S	0.32					
Olsenella; ASV 4	p	ns	< 0.05	<0.0001	ns	ns	ns
	r_S		-0.26	-0.53			
Bifidobacterium; ASV 5	p	ns	ns	< 0.01	< 0.01	ns	ns
	r_S			0.42	0.39		
Pseudoramibacter; ASV 6	p	< 0.0001	< 0.01	<0.05	<0.05	<0.0001	<0.001
	r_S	-0.76	0.36	0.34	0.34	0.62	0.45
[Eubacterium] nodatum group; ASV 7	p	ns	<0.01	<0.001	ns	ns	<0.0001
	r_S		0.36	0.44			0.48
Olsenella; ASV 8	p	<0.001	ns	ns	ns	<0.05	ns
	r_S	-0.45				0.29	
Lactobacillus; ASV 10	p	<0.05	ns	ns	<0.01	ns	ns
	r_S	0.31			-0.39		
Olsenella; ASV 12	p	ns	ns	ns	ns	ns	ns
	r_S						
Acetitomaculum; ASV 13	p	ns	<0.0001	ns	ns	ns	<0.01
	r_S		0.67				0.37
Olsenella; ASV 14	p	ns	ns	ns	ns	ns	ns
	r_S						
Corynebacterium; ASV 15	p	ns	ns	ns	ns	ns	ns
	r_S						
Lactobacillus; ASV 17	p	ns	<0.0001	ns	ns	ns	ns
	r_S		-0.49				
Caproiciproducens; ASV 19	p	ns	<0.05	ns	<0.01	ns	ns
	r_S		-0.29		0.36		

ns, not significant (p>0.05)

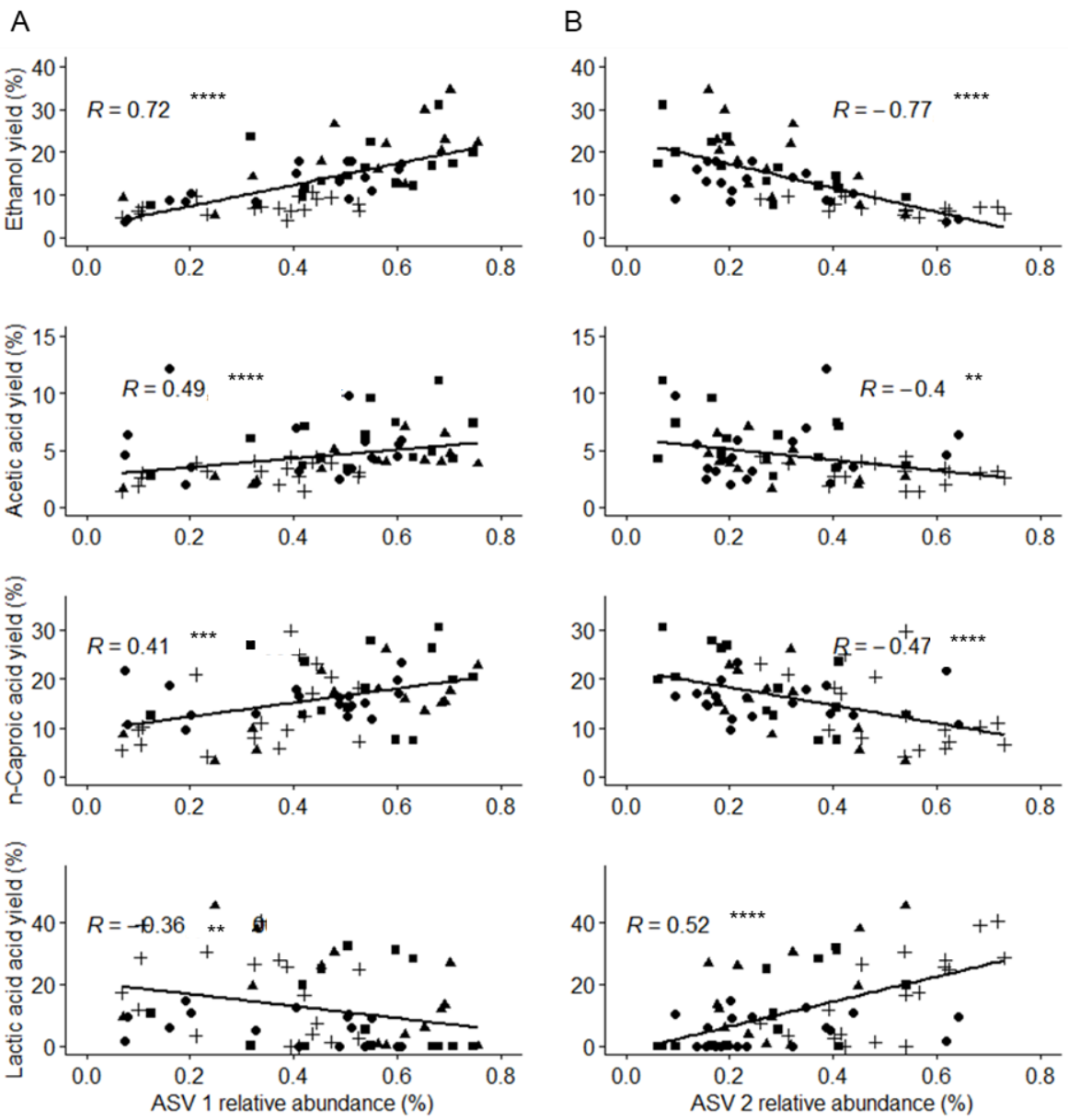


Figure S5-11 *Scatter plots with spearman correlation (R) between the relative abundance of (**A**) Lactobacillus ASV 1 and (**B**) Lactobacillus ASV 2 to ethanol, acetic acid, n-caproic acid and lactic acid yield as indicated by title axis. Symbols represent samples taken from reactors fed bi-weekly (● before 8 HRT of operation, ▲after 8 HRT of operation), daily (+) or switched to bi-weekly feeding after daily feeding for 8 HRT (■). Data Symbols "**", "***" and "****" represent p-values of < 0.01, < 0.001 and <0.0001, corrected for multiple comparisons with a FDR of 5%.*

5.2.7. Cycle study

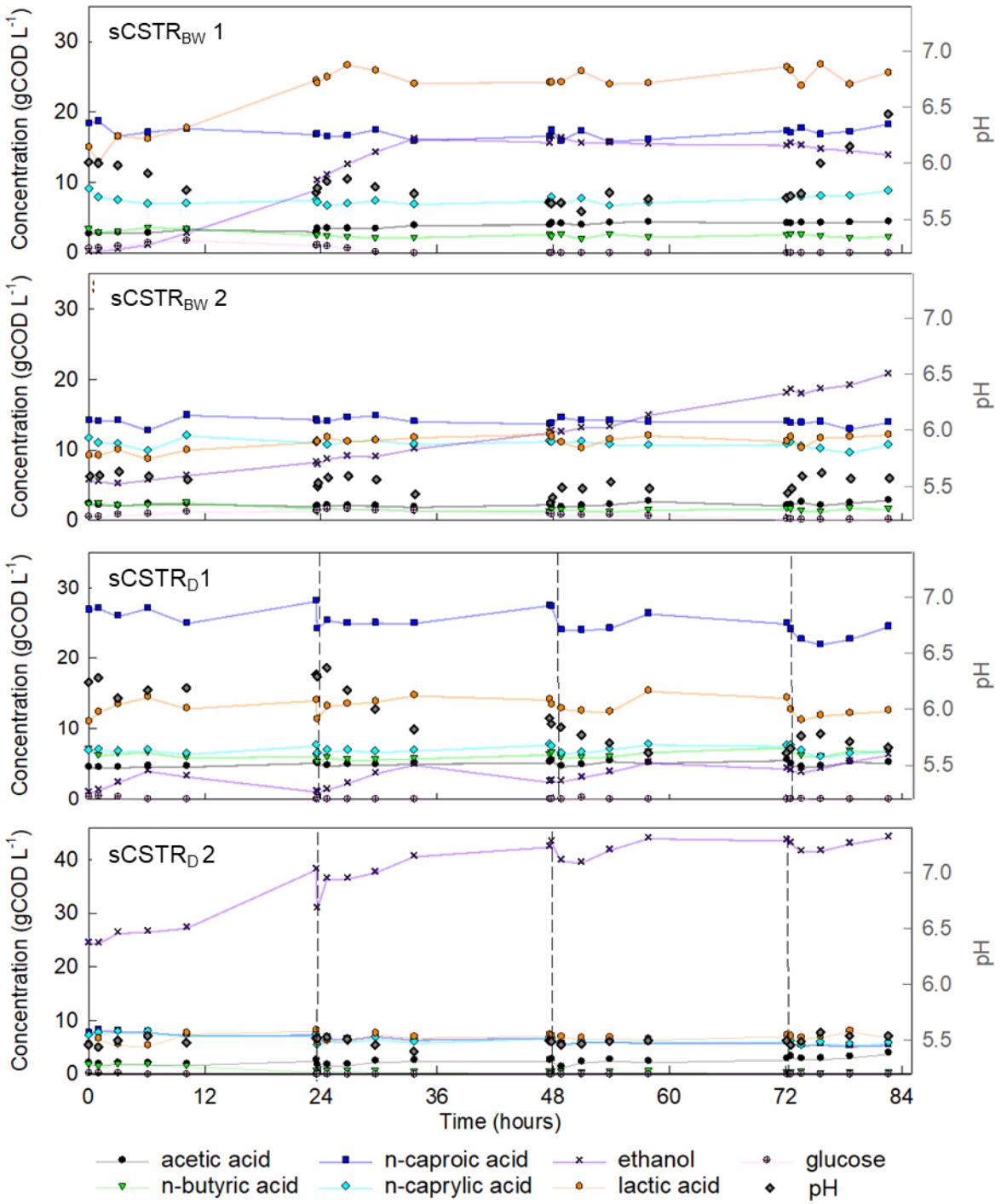


Figure S5-12 *Cycle study performed in between Day 49 to 52.5 of operation. The pH and concentration profiles of fermentation compounds over 84 hours in bi-weekly fed reactors (sCSTR$_{BW}$ 1 and 2) and daily fed reactors (sCSTR$_{D}$ 1 and 2). Time 0 corresponds to a sample taken straight after feed addition and the dashed lines indicate feeding events in the daily fed reactors.*

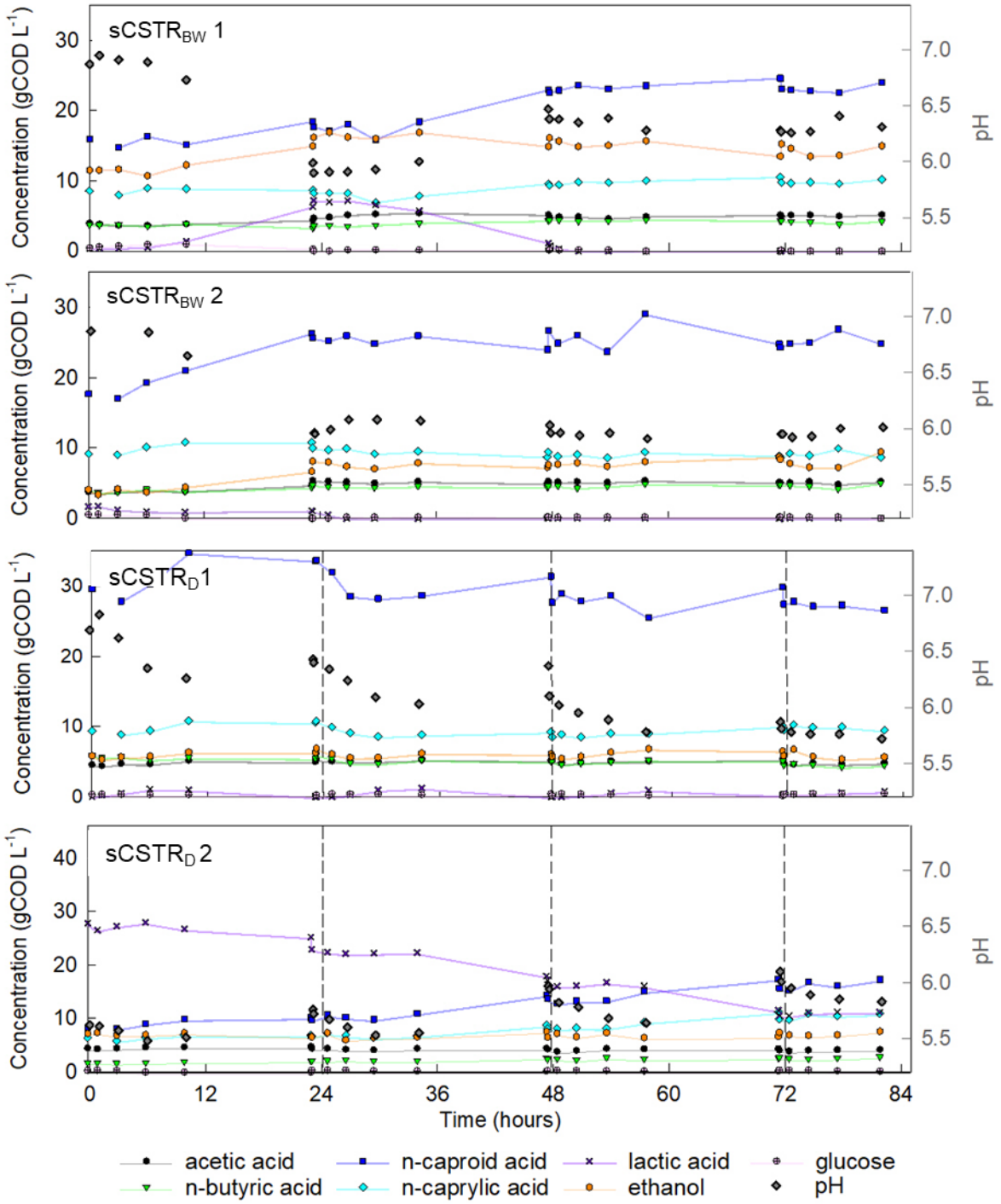


Figure S5-13 *Cycle study performed in between Day 73 to 76.5 of operation. The pH and concentration profiles of fermentation compounds over 84 hours in bi-weekly fed reactors (sCSTR$_{BW}$ 1 and 2) and daily fed reactors (sCSTR$_{D}$ 1 and 2). Time 0 corresponds to a sample taken straight after feed addition and the dashed lines indicate feeding events in the daily fed reactors.*

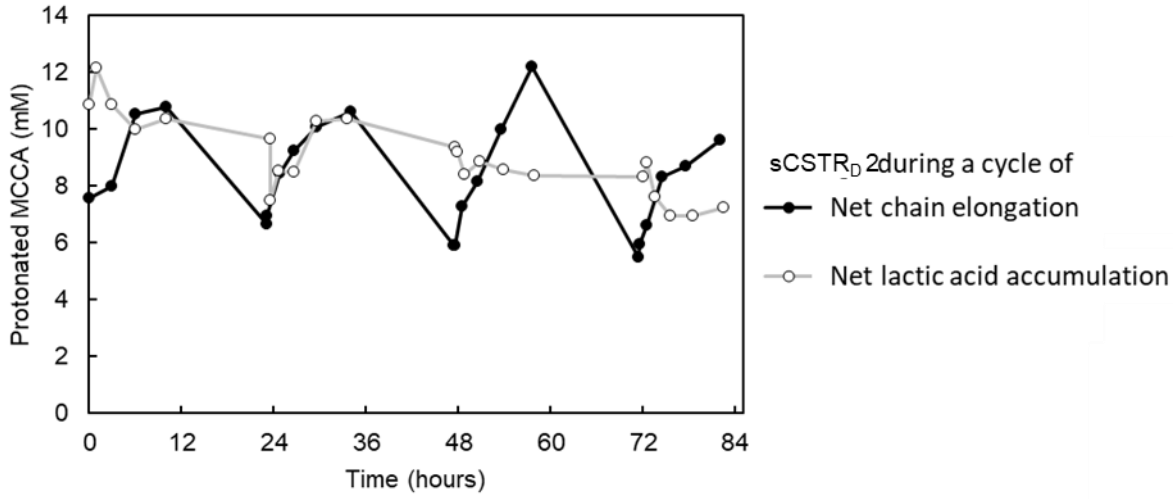


Figure S5-14 *Concentration of protonated MCCA in reactor sCSTR$_D$ 2 during two different fermentation cycles whereby in one lactic acid accumulated and in the other MCCA were produced.*

Chapter 6. The inherent variability of a food waste feedstock and its consequences on acidogenic fermentation

The previous research chapters focussed on the manipulation of operating parameters to steer the outcome in food waste fermentation in line with the second thesis goal (identifying operational strategies for MCCA production from food waste). By working with a real food waste feedstock, variations in organic content and composition are to be expected. As mentioned in the previous chapters, it is necessary to understand how these changes in the feedstock might influence the fermentation process and affect the selection of operating conditions. Therefore, this chapter addressed research objective four on monitoring feedstock composition and its impact on the fermentation process. Batch tests with intermittent sampling and cycle studies were included to characterise the fermentation pathways as outlined by research objective six.

This chapter is submitted as a traditional chapter part of an alternative thesis format in line with Appendix 6A of the “Specifications for Higher Degree Theses and Portfolios” as required by the University of Bath

6.1. Introduction

Food waste (FW) is rich in moisture and nutrients, making it a suitable feedstock for anaerobic biotechnologies such as anaerobic digestion (AD) or the carboxylate platform as a means of treatment and resource recovery. However, FW is a highly varying feedstock, for instance, the composition varies significantly depending on geographical location, source of collection, and there are also seasonal variations [1]. Literature data shows this inherent scatter and generally has high standard deviations. This is true not only for physicochemical characteristics such as total COD (198 ± 89 g kg_{ww}^{-1}), but also for nutritional elements such as total ammonia nitrogen (731 ± 958 mg L^{-1}). It is also the case that differences in pretreatment or storage will affect the feedstock, for example the high biodegradability of FW will cause it to spontaneously ferment during storage [2]. This can be used to an advantage for AD, as the storage conditions and time can be adjusted to enhance hydrolysis and hence increase methane productivity [3].

This complex and variable composition of FW makes it difficult to predict its anaerobic biodegradability [4]. In AD, general day-to-day variations in FW do not compromise methane yields [5, 6]. Neither does pasteurisation, a pretreatment to AD commonly applied to sterilise organic waste containing animal by-products if the digestate is to be used as fertiliser [7]. Song, et al. [8] suggest that the long hydraulic retention time (HRT) used in FW digesters (average of 44 days) is likely to smooth moderate fluctuations in daily loading. However, unexpected sudden increases in organic content of the feedstock can result in an organic overload, and subsequent carboxylate accumulation and process failure [9]. For processes relying on acidogenic fermentation, the retention times are generally much shorter than for AD, ranging from a couple of hours to 16 days, to force carboxylate accumulation [10]. Therefore, a variation in the feedstock or changes in its storage will have a higher impact on process stability in such systems. Acidogenic fermentation has a wider product range compared to AD, including H_2, lactic acid, short chain volatile fatty acids (VFA) or medium chain carboxylic acids (MCCA). Thus, instability of the feedstock quality could change the predominant metabolic pathways and generate different, and undesired, products.

Different liquid FW streams generate different acidogenic fermentation products depending on their sugar, ethanol and lactic acid content [11, 12]. This has been shown for FW feedstocks including carbohydrate, protein or lipid-rich pre-consumer restaurant FW, cheese whey, maize silage, microalgae, fruit pulps, brewery residues, tofu, egg white, olive mill effluent, or winery wastewater [13-18]. Chatellard, et al. [19] noted performance differences due to carbohydrate content. They fermented different model lignocellulosic compounds, from monomers to more complex compounds, and observed that each

influenced acidogenic batch fermentation and the resulting microbial community in a different manner. For acid whey valorisation it has been shown that feedstock composition, i.e., lactose, lactate and ethanol content, influences MCCA production rate [20]. It is generally known that different FW feedstock composition and quality influences acidogenic fermentation outcomes, but there is little information on how variations in solid-rich, mixed FW, such as is used in recycling centres, affect acidogenic fermentation outcome. Research is particularly limited for production of chain elongation products (MCCA). To use acidogenic fermentation of FW in resource recovery at recycling facilities, we need to understand how the inherent feedstock variability affects the product outcome. Therefore, this chapter assesses variations in the feedstock used, and how these affect acidogenic fermentation.

6.2. Material and methods

6.2.1. Food waste collections

Three different types of FW were collected: FW used as feedstock for AD at an industrial recycling plant (FW_{rec}, GENeco, Avonmouth, UK), cafeteria FW (FW_{caf}), and liquid food-processing wastewaters (Table 6-1). Each FW collection underwent chemical analysis (Section 6.2.3.) to determine the feedstock variation. Data was obtained from the full-scale AD plant and from the literature to evaluate the feedstock composition in a wider context.

Eight samples of FW_{rec} were collected, two of which had undergone pasteurisation. The FW_{rec} comprised packaged and unpackaged FW collected from households, supermarkets, catering facilities and restaurants. The FW is unpacked at the plant, and then ground and mixed with liquid streams from food-processing industries, and/or the liquid fraction of AD effluent, to form a slurry. The blended mixture is stored in buffer tanks and pasteurised (70 °C for 60 min) before being fed to mesophilic anaerobic digesters. FW samples were characterised and stored in aliquots (-18 °C) before use in fermentation experiments. Fresh FW_{caf} was obtained from a cafeteria (NTU, Singapore) as the solid cooked food fractions discarded by customers, and hard residuals such as shell and bones were manually removed. It was then suspended in reverse osmosis (RO) water for characterisation and used fresh in fermentation experiments. The liquid food-processing waste streams were from soaking yellow or black soybeans (Ysoy, Bsoy) or brewery cleaning water (Brew)(Singapore). The former was collected from a processing facility where the soybeans are soaked for 4 hours to improve digestibility before further processing. The later was collected from a brewery as the residue from rinsing the fermentation tanks. These FW waters were characterised fresh and stored for less than one day at 4 °C prior to fermentation experiments.

Table 6-1 *Overview of food waste (FW) feedstock collections and their application. Each collection was characterised and summarised in the result section (Table 6-3).*

ID	Date	Location	Info	Application
FW_{rec} 1	09/11/2016	Avonmouth, UK	Non-pasteurised FW from buffer tank	Chapter 3: reactor operation
FW_{rec} 2	06/12/2016		Non-pasteurised FW from buffer tank	Chapter 3: reactor operation
FW_{rec} 3	07/06/2017		Non-pasteurised FW from buffer tank ($FW_{pre\text{-}past}$)	Chapter 6: BMP assay ($FW_{pre\text{-}past}$)
FW_{rec} 4	07/06/2017		Pasteurised food waste ($FW_{post\text{-}past}$)	Chapter 6: BMP assay ($FW_{post\text{-}past}$)
FW_{rec} 5	25/01/2018		Non-pasteurised FW from buffer tank	Chapter 4: Reactors HH/LO
FW_{rec} 6	10/05/2018		Pasteurised FW	Chapter 4: Reactors LH/LO and LH/HO, Chapter 6: kinetic test
FW_{rec} 7	28/01/2019		Blend of non-pasteurised FW collected on 03/12/2018 and 28/01/2019	Chapter 6: kinetic test and sucrose supplementation, long term evaluation of sucrose enrichment
FW_{rec} 8	09/07/2019		Non-pasteurised FW from buffer tank	Chapter 6: AFP assay, kinetic test and sucrose supplementation
FW_{caf}	twice a week from 10/09/2019 to 24/01/2020	Singapore	Cafeteria FW	Chapter 5: reactor operation Chapter 6: AFP assay
Ysoy	weekly from 29/08/2019 to 24/01/2020		Yellow soybean soaking wastewater	Chapter 5: reactor operation Chapter 6: AFP assay
Bsoy 1	05/09/2019		Black soybean soaking wastewater	Chapter 6: AFP assay
Bsoy 2	30/09/2019			
Brew 1	26/09/2019		Brewery cleaning water	Chapter 6: AFP assay
Brew 2	06/11/2019			

BMP, biochemical methane potential; AFP, acidogenic fermentation potential

6.2.2. Experimental setup

6.2.2.1. Anaerobic biodegradability tests

The biochemical methane potential (BMP) of FW_{rec} was determined as a benchmark for the maximum anaerobic biodegradability of the feedstock [21]. The BMP assay was done on FW_{rec} samples pre- and post-pasteurisation ($FW_{pre\text{-}past}$, $FW_{post\text{-}past}$). Fresh digestate from the full-scale AD plant (GENeco, Avonmouth, UK) was collected as inoculum 4 days prior to the test and stored at 4 °C until use (20.9 ± 0.2 g L^{-1} VS). Batch tests were set up using the Bioprocess Control AMPTS II system (Bioprocess Control, Lund, Sweden). This comprises 500 mL reactors submerged in a water bath, sealed with a cap incorporating a vertical stirrer, and connected to a gas volume analyser (Bioprocess Control AB, Lund, Sweden). The reactor working volume was 300 mL. The produced CH_4 volume was measured after CO_2 stripping and reported at Standard Temperature and Pressure (STP, 273.15 K, 100 kPa). Reactors were set up with a food-to-microorganisms (F/M) ratio of 0.5 $gCOD_{fed}$ $gVS_{inoculum}^{-1}$ by diluting the inoculum to 10 gVS L^{-1} in the reactor by addition of tap water. This corresponded to a VS addition per substrate of 3.58 and 3.44 gVS L^{-1} for $FW_{pre\text{-}past}$ and $FW_{post\text{-}past}$, respectively. The pH was corrected to 7 at the start. The BMP reactors were incubated at 35 °C for 30 days. Tests were run in triplicate with a control to correct for the autodigestion capacity of the inoculum.

The BMP values (based on tCOD or VS fed), the anaerobic biodegradability (BD), and estimated kinetic hydrolysis constant (k_h), were determined following Raposo, et al. [22]. BMP was determined from the net experimental methane yield, as the amount of CH_4 produced at STP (corrected for CH_4 production from the controls), normalised for organic material added and expressed per total COD or VS in the feed. BD was calculated as the ratio of the experimental BMP_{COD} over the theoretical maximum BMP (355 mL of CH_4 STP per gCOD fed). The k_h was estimated by fitting the net cumulative methane yield on COD to a first order degradation model (Equation (6-1)), solving for k_h using the Goal Seek function in Excel:

$$B_t = B_o(1 - e^{-k_h t}) \quad (6\text{-}1)$$

Where t is the time (day) and B_t and B_o represent the net cumulative methane yield (mL CH_4 $gCOD^{-1}$, STP) produced at time t and at the end of the assay.

6.2.2.2. Acidogenic fermentation potential tests

Seven-day batch tests of the different types of FW and FW enriched with sucrose were performed to determine the impact of composition on acidogenic fermentation potential

(AFP) (Table 6-2). The AFP of the liquid wastewaters (Ysoy, Bsoy and Brew) were assessed individually and in co-fermentation with FW_{caf}. For co-fermentation, FW_{caf} was blended with food-processing wastewaters in a 1:2 weight ratio. To determine the effect of variations in the feedstock, different collections of the same type of FW were tested. These are indicated via a superscript, e.g., FW_{caf}^{a} and FW_{caf}^{b} are time separated samples of the same waste. Acid inhibition was estimated in two sets of experiments on Days 1 and 3 of fermentation, by measuring and correcting pH if needed (Table 6-2).

Table 6-2 *Specifications of AFP batch tests according to the feedstock tested. Pressure from biogas accumulation was released after Days 1 and 3 of fermentation to measure pH and correct to a minimum of 5.5 if needed.*

Feedstock	F/M	Gas release	pH correction
	$gCOD_{fed}\ gVS_{inoculum}^{-1}$	Yes / No	Yes / No
Pure feedstock			
Bsoy	0.19 ± 0.01	No	No
Ysoy	0.30 ± 0.03	No	No
Brew	0.41 ± 0.02	No	No
FW_{caf}	5.0 ± 0.2	No	No
FW_{rec}	5.08 ± 0.05	No	No
Co-fermentation			
FW_{caf} : Brew	5.0 ± 0.9	No	No
FW_{caf} : Ysoy^{a}	5.0 ± 0.1	No	No
FW_{caf} : Ysoy^{b}	5.0 ± 0.7	Yes	No
FW_{caf} : Ysoy ^{b} - pH	5.0 ± 0.7	Yes	Yes
FW_{rec} : 2%w/w sucrose	5.08 ± 0.04	No	No
FW_{rec} : 5%w/w sucrose	5.08 ± 0.04	No	No
FW_{rec}: 10%w/w sucrose	5.08 ± 0.03	No	No

Batch tests were performed in triplicate in 120 or 140 mL serum flasks with the working volume set at 80 mL by addition of tap water. AD effluent was seeded at 5 $gVS\ L^{-1}$ and feedstock was added to obtain an organic load of 25 $gCOD\ L^{-1}$ to give an F/M ratio of 5, as found to be optimal for acidogenic fermentation with AD effluent as inoculum [12]. If the total COD of the feedstock was not high enough to reach the set organic load, the maximum obtainable F/M was used (Table 6-2). Reactors were seeded with AD effluent from a sewage sludge digester at a wastewater treatment plant (Changi, Singapore) with a pH of 7.6 ± 0.1 and estimated biomass content of 24.5 ± 0.9 $gVS\ L^{-1}$. The FW_{rec} tests were inoculated with AD effluent from FW AD (GENeco, Avonmouth, UK) with a pH of 7.7 and

17.96 ± 0.01 gVS L^{-1} biomass. After collection the digestate was left to degas at 35°C for a minimum of 24 hours to minimize residual organics prior to inoculation.

At the start, pH in the reactors was corrected to between 7.2 and 7.7 and headspaces were flushed with N_2 for 1 minute. Reactors were incubated for 7 days at 35 °C in a water bath or incubator equipped with a horizontal shaking plate. Biogas production was monitored by pressure build-up either determined with a manometer or volume displacement in a syringe, and normalised to STP. Control reactors were run to determine the auto-fermentation of the inoculum. Product yields were determined as the concentrations measured on Day 7, corrected for concentrations on Day 0, divided by the organic content of the feed (expressed in tCOD). Gas production yields were corrected for biogas from the inoculum by subtracting the biogas yield from control reactors. Solids reduction was calculated as the percentage of solids removed relative to the initial solids content (feedstock + inoculum).

6.2.2.3. Kinetic batch tests

Three different FW_{rec} samples (FW_{rec} 6, 7 and 8 in Table 6-1) and two FW_{rec} samples enriched with 2% w/w sucrose (FW_{rec} 6 and 8 in Table 6-1) were fermented in a reactor setup allowing intermediate sampling to track product evolution and evaluate feedstock variation and effect of readily biodegradable sugar. Tests were performed in triplicate in 250 mL glass bottles with the working volume made up to 200 mL by addition of tap water. As with the AFP assays, the pH was corrected to around 7.2 and headspaces were flushed with N_2 for 1 minute on Day 0. Reactors were incubated over 7 days at 35 °C in a water bath equipped with a horizontal shaking plate. Reactors were sealed with modified caps to include a gas and liquid sampling port. Due to limitations in reactor design, gas pressure was released automatically if it exceeded 3.7 bar through the sampling ports opening the headspace to atmosphere until closed again within 12 hours. On Days 1, 2, 5 and 7, biogas production was monitored by volume displacement in a syringe and normalised to STP. After gas pressure was released, samples of the gas and liquid phases were taken, and pH and composition analysed. If the pH was below 5.5 after sampling, NaOH was dosed to increase pH above 5.5 to avoid acid inhibition.

6.2.2.4. Semi-continuous stirred tank reactors

To evaluate the effect of a higher fraction of easily biodegradable sugars in the feedstock on functionality and microbial community enrichment, FW enriched with 2 w/w% sucrose was fermented semi-continuously at long-term (over 7.2 HRT). The semi-continuous stirred tank reactors (sCSTR) were operated in the same way as Chapters 3 and 4 [23, 24]. In summary, two 2 L glass bioreactors were run in duplicate with a 0.6 L working volume,

equipped with mechanical vertical stirrers (Bioprocess Control, Lund, Sweden) and kept at 35 °C. A fixed volume of reactor effluent was replaced every 3.5 days with thawed FW (collection FW_{rec} 7 in Table 6-1) and 2 % w/w of added sucrose to achieve an OLR of 11 ± 1 gCOD $L^{-1}d^{-1}$ and a HRT of 15 ± 1 days. The OLR and HRT were chosen to be similar and higher, respectively, to those used in Chapter 4 for targeted MCCA production (i.e., in comparison to the system labelled HH/LO)[24]. The pH was corrected to 5.9 ± 0.1 with sodium hydroxide (1 or 2 M) each feeding event. Gas production was monitored via water displacement using 2 L graduated glass columns containing acidified water (pH < 4.3, HCl), and normalised to STP. The water columns were connected to the reactor headspace via a buffer bottle, to avoid liquid running back to the reactors at low headspace pressures.

At start-up, reactors were inoculated with enriched acidogenic fermentation culture from an in-house reactor, which had been stored at 4°C [23]. The seeding culture was diluted with tap water to obtain a biomass content of 14 gVS L^{-1}, and left to acclimatise overnight to temperature (35 °C) in a water bath. The first feed was diluted 1:2 with tap water. Day 0 of operation corresponds to the first day of feeding at full organic strength. After operating for three HRT, fermentation cycle studies were performed as per De Groof, et al. [23].

The OLR, HRT, net product yields (Y_p), and extent of acidification were calculated for each feeding cycle, approximated to continuous operation, as per De Groof, et al. [23]. The average OLR and HRT were calculated over the entire operation for the two duplicate reactors. Statistical analysis was performed using R v.4.0.0 through the Rstudio IDE using the R Stats package v.3.6.2 [25]. Correlations in fermentation product yield and sodium hydroxide dosing were determined using the Spearman's rank correlation coefficient (r_S), and visualised in a matrix of correlation coefficients using the *Hmisc* (v4.4-1) and *corrplot* (v0.84) packages [26, 27]. The p-values resulting from the correlation analysis were corrected for multiple comparisons using a false discovery rate (FDR) of 5% [28].

Biomass samples were taken to study the microbial community enrichment using 16S rRNA gene amplicon sequencing, targeting the bacterial and archaeal variable region V4 (Section 6.2.4). Duplicate samples were taken from both reactors on Day 0 for inoculum characterisation. Duplicate samples were taken during operation at the peak of caproic acid production (Day 28) and during a peak in lactic acid production (Day 94 Reactor 1, Day 70 Reactor 2) to evaluate the microbial communities.

6.2.2.5. Phase separation of MCCA

During two of the FW fermentations, floating oil and grease-like layers were observed in the reactors and at the top of samples. The layers were analysed for the presence of carboxylic

acids to evaluate the potential for phase separation of protonated carboxylic acids. The samples were left to settle, and the immiscible top layer was pipetted into a separate vial. Carboxylic acid concentrations were measured by HPLC-RI or GC-FID (see Section 6.2.3.). Enrichment was determined as the concentration in the oil phase relative to the concentration in the whole sample.

Insoluble top layers with a more solid consistency were harder to separate manually, so the carboxylic acid concentration was measured in the whole sample (C2-C6 following GC-FID, Section 6.2.3.) to obtain the total, bulk concentration (C_{tot}). Then the same sample was weighed (m_{tot}) and centrifuged (15min × 4.500g) to separate a top floating layer (Phase A), the liquid supernatant (Phase B), and the solid bottom (Phase C). Each of these layers was weighed ($m_{A,B\ or\ C}$) and the concentration of carboxylic acids ($C_{A,B\ or\ C}$) was measured in each of them. By converting the concentrations in each phase to mass it was possible to estimate the overall concentration resulting from measuring each phase separate (C_{phases}), which allowed comparison against C_{tot} to evaluate mass balance. See Equations (6-2) and (6-3).

$$C_{phases=} \frac{m_A C_A + m_B C_B + m_C C_{AC}}{m_{tot}} \quad \text{(gCOD kg}^{-1}\text{)} \quad (6\text{-}2)$$

$$Rel.\,mass\ balance = \frac{\sqrt{(C_{tot} - C_{phases})^2}}{Average(C_{tot}, C_{phases})} \quad (\%) \quad (6\text{-}3)$$

6.2.3. Chemical analysis

Total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined using fresh samples according to Standard Methods 2540 D and E, and expressed on a fresh weight basis [29]. The total chemical oxygen demand (tCOD) represents the content of both particulate and soluble matter. The soluble COD (sCOD) was measured from filtered samples (0.45 µm). Chemical oxidation of the organic content in the samples was performed in cuvette tests (LCK014, 1-10 $g_{COD}\,L^{-1}$ and LCI400, 0-1 $g_{COD}\,L^{-1}$ or Cat. 2125915 Hach, Düsseldorf, Germany), based on a dichromate oxidation method (5220-C and D, [29]), and measured with a Hach DR 2800 Spectrophotometer.

C1-C5 n-carboxylic acids, ethanol, lactic acid, and glucose were measured by HPLC-RI using an adapted method from Coma, et al. [12], with the oven temperature adjusted to 65 °C. The more hydrophobic MCCA (C5-C8), and all C2-C8 n-carboxylic acids for phase separation tests, were analysed by GC-FID as per Chapters 3 and 4 following the method adapted from Manni and Caron [30].

Gas samples were taken for composition analysis (N_2, O_2, H_2, CH_4 and CO_2) from reactor headspaces in the batch tests, or from the glass gas collection columns in the semi-continuous reactors, immediately before sample withdrawal and feed addition. For the anaerobic biodegradability tests (Section 6.2.2.1.) and sCSTRs (Section 6.2.2.4.) gas composition was analysed using two systems: GC-FID/TCD for CH_4 and CO_2 quantification, and GC-TCD for H_2, N_2 and O_2, see Chapter 3 and 4 [23, 24]. During AFP tests fermenting FW_{caf}, Ysoy, Bsoy, Brew or mixtures thereof, gas composition was determined by GC-TCD, see Chapter 5. Gas composition for the remaining AFP and kinetics tests was measured by GC-TCD (Trace 1300, ThermoFisher Scientific, MA, USA) equipped with split/splitless injector module, micropacked Shincarbon-ST column (ShinCarbon ST, 100/120 mesh, 2 m, 1/16 in. OD, 1.0 mm ID (Cat.# 19808)). Samples were injected at 0.50 µL with split ratio of 2 and inlet temperature of 150 °C. Argon was used as carrier gas at constant total flow rate of 10.0 ml min^{-1} with oven temperature at 40 °C for 4.00 min, then ramped at 120 °C min^{-1} to 100 °C and held for 2.50 min. Gases were detected by TCD at 280 °C and filament temperature of 380 °C and reference gas flow at 1.0 mL min^{-1}. Quantification was achieved by one-point calibration forced through 0 with a 10 volume% calibration mixture (10% H_2, 10% CO_2, 10% CH_4, β-grade, BOC, Surrey, UK) and air (21% O_2, 78% N_2).

6.2.4. Microbial community analysis

Samples were stored at -18°C before sending to DNAsense (Aalborg Øst, Denmark) for DNA extraction, library preparation, 16S rRNA gene amplicon sequencing and bioinformatics processing. This included clustering reads by operational taxonomic units (OTUs) at 97% similarity and assigning taxonomy using the SILVA database, as per De Groof, et al. [23]. The results were analysed using the DNAsense app (DNAsense, Aalborg Øst, Denmark), which is based on the *ampvis* package (v.2.5.8) in R (v. 3.5.1) [25, 31]. The number of sequencing reads per sample was between 17579 and 25383 and rarefaction curves were generated (Figure 6-1). To allow linear comparison of community structures, effective alpha-diversity, i.e., 1st and 2nd Hill number (^1D and ^2D), were calculated from the common alpha-diversity measures available from the DNAsense app [32-34].

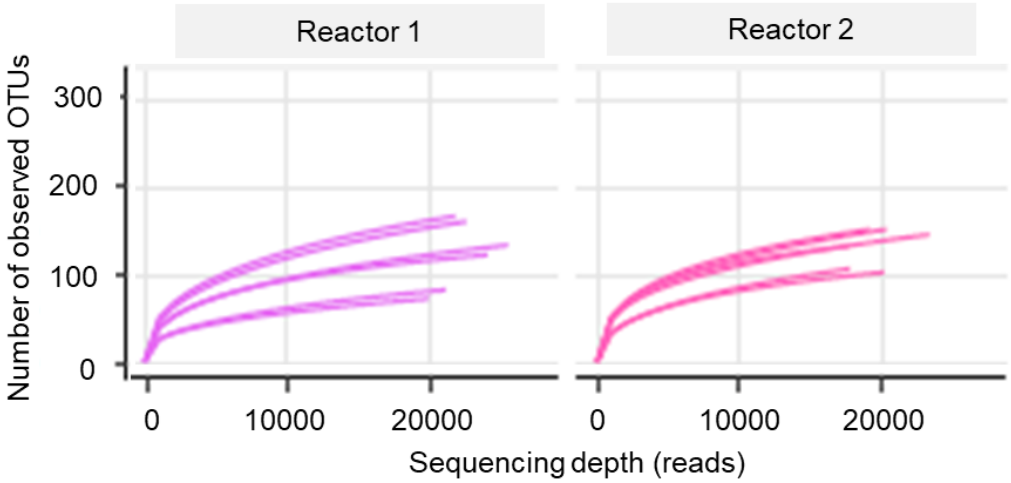


Figure 6-1 *Rarefraction curves for reactor biomass samples calculated as the number of observed OTUs (Species) as function of the sequencing depth. Flattening of the curve indicates exhaustive sequencing of the diversity in the samples. Two reactors were operated in duplicate and 3 timepoints were sampled in duplicate, i.e. a total of 6 biomass samples per reactor.*

6.3. Results and discussion

6.3.1. Variations of the food waste feedstock

The composition of the different FW samples collected were characterised to determine natural variability (Table 6-3). Compositions per type of FW feedstock were averaged to cross reference with studies (Table 6-4). Previous characterisation of the mixed FW from the full size AD plant (GENeco, Avonmouth) over 2 years indicated a highly varying feedstock with TS ranging from 5.5 to 18.5 %, tCOD from 69.2 to 264 g_{COD} kg_{ww}^{-1}, and VFA 2.1 to 11.3 g L^{-1} VFA (internal report). Most FW samples from this AD plant (GENeco, Avonmouth) in this work fall within that range (FW_{rec} in Table 6-3). The second solid FW used in this work, FW_{caf}, had solids and tCOD contents that were nearly three times higher than FW_{rec}. By contrast, the liquid FW waters from soybean processing and brewery cleaning had a much lower organic content.

Table 6-3 *Overview of feedstock characteristics of the different types of food waste (FW) collected (location, time of collection and for which study these feedstocks were used are available in Table 6-1). Chemical analysis was performed in duplicate unless specified otherwise (^{n}). *Where VS or TS could not be determined because the feedstock was too dilute, the VSS and TSS were reported instead. FW_{rec}: feedstock from an industrial AD plant (GENeco, Avonmouth), FW_{caf}: cafeteria FW, Y/Bsoy: yellow and black soybean soaking water, Brew: brewery wash water.*

	pH	Conductivity	TS (*TSS)	VS (*VSS)	tCOD	sCOD	Lactic acid	Ethanol	VFA (C1-C4)	Glucose
		mS cm^{-1}	% w/w (*g L^{-1})	% w/w (*g L^{-1})	g_{COD} kg_{ww}^{-1}	g_{COD} kg_{ww}^{-1}	g_{COD} kg_{ww}^{-1}	g_{COD} kg_{ww}^{-1}	g_{COD} kg_{ww}^{-1}	g_{COD} kg_{ww}^{-1}
FW_{rec} 1	4.99 ± 0.08	NA	9.94 ± 0.07	8.83 ± 0.00	150 ± 1	38 ± 1	7.8 ± 0.4	3 ± 2	2.7 ± 0.2	3.9 ± 0.2
FW_{rec} 2	5.30 ± 0.06	6.3 ± 0.2	18 ± 2	16 ± 1	297 ± 9	38 ± 0.1	2.65 ± 0.02	1.7 ± 0.3	2.6 ± 0.6	5.4 ± 0.9
FW_{rec} 3	4.03$^{n=1}$	8.6 $^{n=1}$	10.2 ± 0.1	9.27 ± 0.1	129 ± 7	21.4 ± 0.5	NA	NA	NA	NA
FW_{rec} 4	4.48 $^{n=1}$	8.8 $^{n=1}$	12.79 ± 0.01	9.59 ± 0.08	139 ± 23	44.3 ± 0.3	NA	NA	NA	NA
FW_{rec} 5	4.1 ± 0.1	6.9 ± 0.2	10.7 ± 0.7	8.8 ± 0.3	130 ± 9	63.7 ± 0.8	21 ± 1	27 ± 1	7 ± 2	1.5 ± 0.3
FW_{rec} 6	4.07 ± 0.02	8.3 ± 0.4	11.3 ± 0.2	10.3 ± 0.2	163 ± 2	76 ± 2	23.1 ± 0.2	10.1 ± 0.8	3.7 ± 0.9	0.1 ± 0.1
FW_{rec} 7	4.02 ± 0.01	8.7 ± 0.2	9.4 ± 0.2	8.5 ± 0.2	146 ± 6	62 ± 3	20 ± 7	16.0 ± 0.3	7 ± 1	0.00 ± 0. 00
FW_{rec} 8	3.79 ± 0.02	9.73 ± 0.08	10.3 ± 0.2	9.3 ± 0.1	147 ± 1	53 ± 12	30 ± 6	7.0 ± 0.9	4.7 ± 0.7	0.00 ± 0.00
FW_{caf} 1	6.31 ± 0.01	NA	30.3 ± 0.4	29.65 ± 0.01	409 ± 11	136 ± 1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.99 ± 0.01
FW_{caf} 2	5.92 ± 0.05	NA	31.06 ± 0.05	30.25 ± 0.01	420 ± 14	155.8 ± 0.8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.38 ± 0.01
FW_{caf} 3	6.22 ± 0.00	NA	28.65 ± 0.07	27.79 ± 0.07	406 ± 17	168 ± 1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.99 ± 0.09
Ysoy 1	5.88 ± 0.01	NA	*0.06 ± 0.01	*0.035 ± 0.001	1.54 ± 0.06	1.44 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.00
Ysoy 2	6.01 ± 0.02	NA	*0.06 ± 0.04	*0.043 ± 0.001	1.39 ± 0.06	1.42 ± 0.02	0.00 ± 0.00	0.17 ± 0.00	0.00 ± 0.00	0.26 ± 0.00
Bsoy 1	6.16 ± 0.02	NA	*0.028 ± 0.000	*0.028 ± 0.000	0.67 ± 0.02	0.65 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.00
Bsoy 2	6.68 ± 0.01	NA	*0.024 ± 0.002	*0.024 ± 0.002	1.19 0.04	1.16 ± 0.01	0.00 ± 0.00	0.08 ± 0.00	0.00 ± 0.00	0.10 ± 0.01
Brew 1	10.99$^{n=1}$	NA	*0.433 ± 0.001	*0.409 ± 0.001	4.31 ± 0.01	3.58 ± 0.03	0.00 ± 0.00	0.09 ± 0.05	0.00 ± 0.00	0.30 ± 0.00
Brew 2	9.16 ± 0.03	NA	*0.70 ± 0.04	*0.67 ± 0.04	2.5 ± 0.1	2.07 ± 0.03	0.00 ± 0.00	0.66 ± 0.06	0.00 ± 0.00	0.00 ± 0.00

NA, not available; ww, wet weight

Table 6-4 *Comparison of food waste parameters used as feedstock in acidogenic fermentation studies.*

Type of FW	pH	TS % w/w	VS % w/w	tCOD $g_{COD}\ kg_{ww}^{-1}$	sCOD $g_{COD}\ kg_{ww}^{-1}$	Reference
Recycling centre (FW_{rec})	4.3 ± 0.5	12 ± 3	10 ± 3	163 ± 55	50 ± 18	this study
Cafeteria (FW_{caf})	6.1 ± 0.2	30 ± 1	29 ± 1	412 ± 13	153 ± 14	this study
Recycling centre	4.6	10.5 ± 0.0	9.6 ± 0.1	157 ± 11	NA	[12]
Recycling centre	NA	20.0 ± 0.2	17.9 ± 0.1	277 ± 53	125.2 ± 0.6	[38]
Cafeteria FW	NA	20 ± 1	19.4 ± 0.3	NA	NA	[39]
Restaurant FW	3.6 ± 0.3	28 ± 2	26 ± 2	376 ± 51	NA	[35]
OFMSW	NA	37.1 ± 0.1	24 ± 4	333 ± 25	NA	[40]
OFMSW	7.7 ± 0.8	NA	NA	380 ± 22	NA	[17]
OFMSW	NA	45 ± 2	22 ± 2	204 ± 29	NA	[41]
Simulated FW	4.1 ± 0.1	6 ± 1	5.5 ± 0.2	NA	NA	[42]
Vegetable and salad waste	4.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	57 ± 4	28 ± 5	[43]
Acid whey wastewater	4.7	5.8 ± 0.2	5.1 ± 0.1	72 ± 0.2	71 ± 0.1	[14, 44]

NA, not available; ww, wet weight

Different FW feedstocks have been used in acidogenic fermentation studies that reported n-caproic acid production (Table 6-4). Comparing these to the feedstock in this work shows that FW_{rec} had a slightly lower solid and COD content, whereas FW_{caf} was generally similar. This difference is due to processing of FW_{rec} within the industrial AD plant, where it is mixed with a liquid stream to obtain a pumpable mixture. It can therefore be expected to have a lower solids content and lower or higher COD content depending on the liquid used for mixing. One of the eight FW_{rec} samples stood out as it had a high tCOD of 297 ± 9 gCOD kg_{ww}^{-1} and TS of 18 ± 2 % (FW_{rec} 2 in Table 6-3). According to site operators, the high COD content could have resulted from mixing the solid FW with oily washings from savoury snack manufacturing. Overall, the liquid used to dilute solids in the AD plant varied from recycled liquors from digestate dewatering, to food processing liquids including waste streams from fruit juice, cheese, potato, ice cream or meat processing, and washing waters from juice, wine, beer, or cider production. Acidogenic fermentation studies with a feedstock with higher solid and COD content than FW_{rec} either diluted the feed to allow operation in stirred tank reactors or used leach bed reactors [35-37]. FW_{caf} was mixed with yellow soybean processing wastewater when used as feedstock for semi-continuous fermentation in a stirred tank configuration (Chapter 5). Mixing of solid and liquid FW streams could lead to significant variation in feedstock characteristics if the liquid used is highly concentrated in dissolved organics. On the other hand, careful blending or appropriate selection of the liquid

source gives the opportunity to control COD content. This requires improved stream characterisation, and hence increases process complexity, but it leads to potentially enhanced and consistent process performance. Further work should determine how to obtain an optimal blend and the practical feasibility of the whole process.

6.3.2. The high anaerobic biodegradability of food waste

An initial biochemical methane potential (BMP) assay was carried out on the feedstock to have an indication of the maximum anaerobic biodegradability (BD) and rate of biodegradation (kinetic hydrolysis constant, k_h). The assay evaluated FW that was not pasteurised ($FW_{pre\text{-}past}$, FW_{rec} 3 in Table 6-1) and pasteurised FW ($FW_{post\text{-}past}$, FW_{rec} 4 in Table 6-1). There was no significant difference in BD and k_h between the FW pre- and post-pasteurisation samples (Figure 6-2), and values were in the upper range of those in the literature (Table 6-5). Within 6.5 days of fermentation 95% of the total biodegradable matter is digested at an estimated k_h of 0.46 day^{-1}. This high biodegradability and rapid digestion rate justifies the use of FW as feedstock for valorisation in anaerobic fermentation processes. Although the FW_{rec} feedstocks are within the range identified for the AD plant, extrapolating the results as a general for FW_{rec} is speculative as its composition varies. Nevertheless, these results confirm the high and rapid biodegradability of the feedstock.

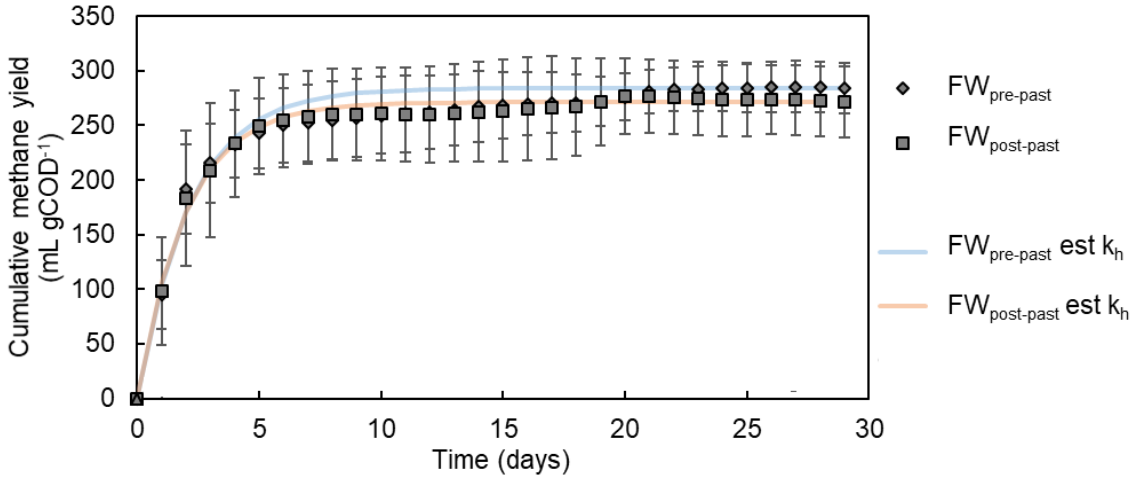


Figure 6-2 *Cumulative methane yield from the BMP assay and fitted curves following a first order degradation solved for the estimated kinetic hydrolysis constant (k_h).*

Table 6-5 *Biochemical methane potential (BMP), anaerobic biodegradability (BD) and kinetic hydrolysis constant (k_h) of food waste (FW) collected from an industrial anaerobic digestion plant before ($FW_{pre\text{-}past}$) and after ($FW_{post\text{-}past}$) pasteurisation (60 min, 70°C), compared to literature data.*

Feedstock		**BMP_{COD}** $mL_{CH_4}\ g_{COD,fed}^{-1}$	**BMP_{VS}** $mL_{CH_4}\ g_{VS,fed}^{-1}$	**BD** %	**k_h** Day^{-1}	**Reference**
$FW_{pre\text{-}past}$	(avg ± sd)	284 ± 23	396 ± 32	80 ± 6	0.46	this study
$FW_{post\text{-}past}$	(avg ± sd)	271 ± 35	394 ± 47	76 ± 9	0.50	this study
3 types of FW	(range)	NA	372 – 421	67 – 91	NA	[4]
10 types of FW	(range)	NA	99 – 645	NA	NA.	[45]
11 types of FW	(range)	NA	165 – 496	56.1 – 99.1	0.26 - 0.64	[46]
FW and OFMSW, pasteurised and non-pasteurised	(range)	NA	330 – 475	86 – 93.7	NA	[7]

NA, not available

6.3.3. The acidogenic fermentation of different food waste feedstock

The acidogenic fermentation potential (AFP) of different types of FW feedstock, individually and in combination, were determined in 7-day batch tests with (i.e., kinetic tests) or without intermittent sampling. The AFP tests were performed on different samples of the same FW source to characterise the effect of inherent feedstock variations. Results of the AFP assays and kinetic tests were summarized in Figure 6-3.

6.3.3.1. Type of food waste determines type of acidogenic fermentation

The COD content of the brewery wastewater (Brew) and the soybean wastewater (Ysoy, Bsoy) was too low to achieve a F/M ratio of 5 $gCOD_{fed}\ gVS_{inoculum}^{-1}$. The low F/M (< 1 $gCOD_{fed}\ gVS_{inoculum}^{-1}$) prevented inhibition of methanogenesis and no acidogenic fermentation products accumulated (Figure 6-3 A).

For the AFP tests with solid FW (FW_{caf} or FW_{rec}), there was enough COD to operate at a F/M of 5 $gCOD_{fed}\ gVS_{inoculum}^{-1}$, and hence inhibit methanogenesis by organic overload. A different fermentation outcome was obtained for each type of FW. Fermentation of FW_{caf}, or co-fermentation of FW_{caf} with one of the liquid wastewaters at a 1:2 weight ratio, resulted in a total yield of liquid products of up to 73 ± 3 % ($gCOD_{liquid\ products}\ gCOD_{fed}^{-1}$) and accumulation of predominantly lactic acid and ethanol up to 10.0 ± 0.3 gCOD L^{-1} and 6.6 ± 0.4 gCOD L^{-1}, respectively. (Figure 6-3 B). During this lactic acid-type fermentation the pH dropped to between 3.65 and 3.94, i.e., around the pKa of lactic acid (3.86, 25 °C). This is in line with some previous reports on FW fermentation where acidification occurred within 2 days via lactic acid accumulation in the absence of pH control [47].

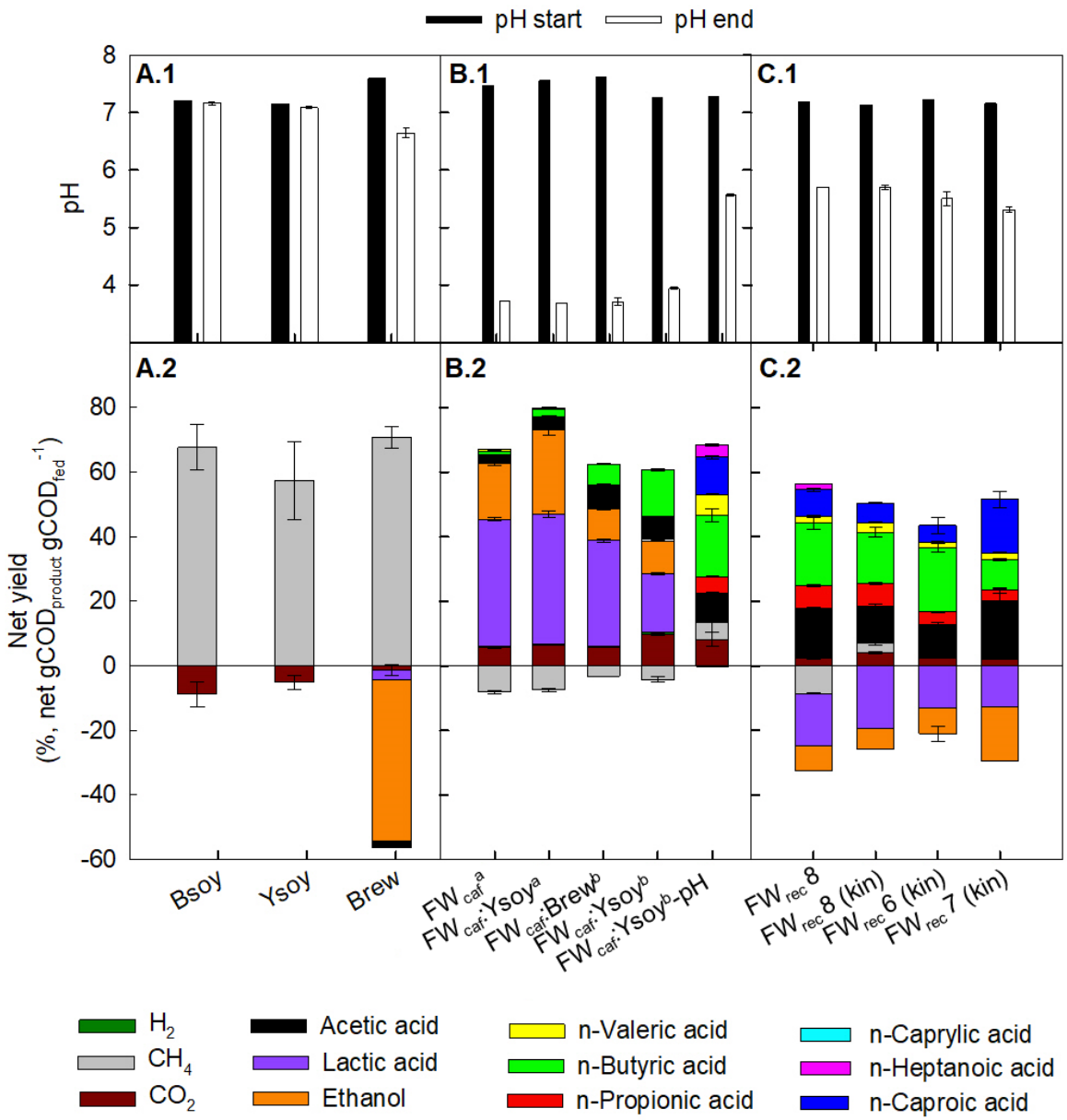


Figure 6-3 *Acidogenic fermentation potential of the different feedstock collected: (**A**) wastewaters from yellow and black soybean processing (Ysoy, Bsoy) and brewery cleaning water (Brew); (**B**) cafeteria food waste (FW_{caf}) and the effect of minimal pH control and; (**C**) food waste collected from a full-scale AD plant (FW_{rec}, numbered according to Table 6-1). The top part shows the pH change and the bottom the net product yields. Tests with intermittent sampling were indicated by "(kin)" as these might underestimate gas production due to gas release at overpressure (3.7bar). Negative yields for biogas compounds indicate less gas was produced than in the accompanying blank assay. Negative yields for liquid compounds indicate they were net consumed from the feedstock.*

Lactic acid production is generally the predominant fermentation type in microbial communities when high concentrations of easily biodegradable compounds are present [48]. By contrast, the total yield of liquid products for fermentation of FW_{rec} (57 ± 2%) was lower than for FW_{caf}. Carboxylic acids were the main liquid products (total 14.2 ± 0.5 gCOD L^{-1}), with 2.1 ± 0.1 gCOD L^{-1} as C6. The final pH in fermentation of FW_{rec} remained above 5.3, higher than for FW_{caf} (Figure 6-3 C). This may not relate to differences in buffer capacity between FW_{rec}, and FW_{caf}, but to their storage before fermentation. The FW_{rec} is usually

collected, transported, blended with water or food-processing wastewaters, and stored over several days in the AD plant. By contrast, FW_{caf} was freshly prepared within 24 hours from post-consumer food scraps. Spontaneous hydrolysis and acidification by indigenous lactic acid bacteria occurs during FW storage [49]. Thus, FW_{rec} generally undergoes more acidogenic fermentation prior to its use, thus lowering the amount of readily available organic content when fed to a reactor. This was reflected in the characteristics of the feedstock, as the pH was lower in FW_{rec} than FW_{caf}, and the presence of higher concentrations of primary fermentation products such as VFA, lactic acid and ethanol (Table 6-4). Therefore, if the target is to produce lactic acid, fresh FW should be used as reactor feedstock or stored in a way to maintain freshness, e.g., chilling. However, minimising degradation during storage might prove challenging at industrial scale, where large volumes of waste from different locations must be collected prior to use. By contrast, pre-fermentation during storage is beneficial for targeting carboxylic acid products and even chain elongation for MCCA production. For AD in general, 5 to 7 days of FW storage are recommended to improve the digestibility, as hydrolysis of solids into soluble organics may be enhanced [3].

6.3.3.2. pH control alters products of cafeteria food waste in batch fermentation

Although lactic acid bacteria have been shown to operate at pH values as low as 3.5 (Itoh et al., 2012), acid inhibition can compromise hydrolysis and overall fermentation yields (Gänzle, 2015; Tang et al., 2017; Wu et al., 2015). Acid inhibition was evaluated for $FW_{caf}{}^{b}$:$Ysoy^{b}$ by adding a test condition where pH was increased if it dropped below 5.5 on Days 1 and 3 of fermentation (marked as $FW_{caf}{}^{b}$:$Ysoy^{b}$ – pH in Figure 6-3 B). Only Day 1 required pH correction from 4.39 ± 0.03 to 6.4 ± 0.3 by NaOH addition. The pH on Day 3 was 5.8 ± 0.1 for $FW_{caf}{}^{b}$:$Ysoy^{b}$ – pH and 3.81 ± 0.00 for the test without pH control. The pH decreased to 5.57 ± 0.02 by Day 7 but was again significantly higher compared with tests without pH control (3.94 ± 0.02).

When pH was controlled the product spectrum mainly comprised carboxylic acids (total of 14.0 ± 0.6 gCOD L^{-1}), including chain elongation products up to C6 (2.9 ± 0.1 gCOD L^{-1}) and C8 (0.28 ± 0.01 gCOD L^{-1}), without any lactic acid or ethanol. This is a product profile more similar to fermentation of FW_{rec}. Lactic acid is converted to mainly acetic and butyric acid in FW fermentation when the pH is kept above 5, or sufficient buffering capacity is provided (Gu et al., 2018; Tang et al., 2017). Therefore, due to the acidogenic nature of the hydrolysis and primary acidogenic fermentation of cafeteria FW, pH should be maintained above a minimum if carboxylic acids are targeted as main products. Methanogenesis is less

inhibited as 1.3 ± 0.5 L L^{-1} of CH_4 was produced in reactors with NaOH addition, which was more than double that produced without pH control (0.46 ± 0.06 L L^{-1} CH_4). Despite the loss of organic to methane, more of the feed COD was converted into liquid products when pH was corrected (56 ± 2%) compared to the tests without pH control (50.5 ± 0.5%). Thus, pH control improved overall fermentation. If lactic acid is targeted as main product, yields could be further optimised by starting fermentation at lower pH and shortening fermentation time to prevent lactic acid conversion [50].

6.3.3.3. Inherent food waste variations affect acidogenic fermentation in batch

FW_{caf}:Ysoy and FW_{rec} samples from different collection times were subjected to an AFP test to evaluate how inherent feedstock variations affect acidogenic fermentation.

For FW_{caf}:Ysoy, the main difference was lower overall liquid product yields for $FW_{caf}{}^{b}$:$Ysoy^{b}$ (50.5 ± 0.4 %) with more C4 (14.4 ± 0.2%) and more biogas (16.0 ± 0.3% COD_{gas}/COD_{fed}) in comparison with $FW_{caf}{}^{a}$:$Ysoy^{a}$ (73 ± 3 % Y_{total}, 2.3 ± 0.0% Y_{C4}, 9.4 ± 0.4% biogas) (Figure 6-3 B). This could be caused by natural heterogeneity and variations in the feedstock, for instance $FW_{caf}{}^{a}$:$Ysoy^{a}$ had a larger fraction of soluble organics (sCOD/tCOD = 41 ± 2 %) compared to $FW_{caf}{}^{b}$:$Ysoy^{b}$ (26 ± 4 %).

The three different samples FWrec were subjected to a 7-day acidogenic fermentation potential (AFP) test (FW 6, 7 and 8 in Table 6-1), with intermittent sampling to evaluate the effect of variations on fermentation mechanism. The three samples of FWrec had similar COD and solid contents ranging between 146 and 163 gCOD L-1 and 9.4 and 11.3 g L-1 TS, respectively. Ethanol and lactic acid concentrations showed the largest variation, ranging from 7 to 16 gCOD L-1 for ethanol and 20 to 30 gCOD L-1 for lactic acid. This could be due to pre-fermentation during storage or from the industrial liquid streams used to dilute the FW within the facility. Regardless of the differences in the feedstock collected, the final product profile and pH of the AFP tests were comparable, and the overall carboxylic acid production was essentially the same (Figure 6-3 C).

Three stages of fermentation could be distinguished in the tests: 1) oxidation of easily accessible compounds and hydrolysis; 2) primary fermentation of hydrolysis products to VFA and ethanol; and 3) formation of chain elongation products (Figure 6-4). In the first 24 hours, biogas production peaked and exceeded pressure limits on the sampling system (around 3.7 bar) resulting in the reactors headspace opening to atmosphere. Residual biogas composition included 3 to 9 % H_2, with CO_2 up to 66%, and CH_4 up to 17%. During this biogas production phase, net lactic acid concentrations dropped to 0, and ethanol decreased below 1 gCOD L^{-1} for all FW, regardless of the difference in initial concentrations

of these compounds. The average pH dropped from 7.2 to between 6.0 and 6.3. This activity, especially H_2 and CO_2 generation, is indicative of hydrolytic fermentative bacteria and syntrophic acetogenic bacteria consuming the soluble compounds, as expected with an inoculum sourced from AD [51-53]. In a second phase of fermentation (Day 1 to Day 5), the predominant liquid products were acetic acid (C2) and C4, with minor n-propionic acid (C3) production. Ethanol was net produced for two of the three FW substrates. The H_2 content in the biogas decreased, whereas CH_4 increased to 28%, which is indicative of hydrogenotrophic methanogenesis. Acidogenesis continued as the average pH dropped to 5.74 ± 0.04. This agrees with what Zhou, et al. [54] described as a mixed-acid metabolic pathway typical for some types of FW fermentation. In a third phase, ethanol eventually decreased for all FW reactors, coupled with an increase in n-caproic acid (C6), and indicating chain elongation via *in situ* production of ethanol. The final MCCA yield (Y_{MCCA}) from fermentation of FW_{rec} 7 was double that of FW_{rec} 6 and FW_{rec} 8 (Table 6-6). The initial ethanol concentration was higher for FW_{rec} 7 and was not completely removed when ethanol production started, contributing to a higher product accumulation. To a lesser extent, FW_{rec} 6 behaved the same way. Therefore, higher initial concentrations of ethanol promoted chain elongation. The differences in initial lactic acid concentration did not appear to affect product outcome, as it was completely removed during oxidation, and no lactic acid was observed in the second phase.

Thus, for batch operation the minor variations seen in FW_{caf}:Ysoy and FW_{rec} resulted in slight differences in fermentation outcome (Table 6-6). However, the predominant fermentation pathways occurred in a similar manner for all three collection of FW_{rec}. It is unlikely that these small variations in feedstock affect long term operation, as they are normally smoothed out during large scale, continuous operation [2]. However, evaluating the range at which these variations occur with an acclimated microbial community, until they start affecting long-term, continuous acidogenic fermentation could reveal approaches to improve process stability.

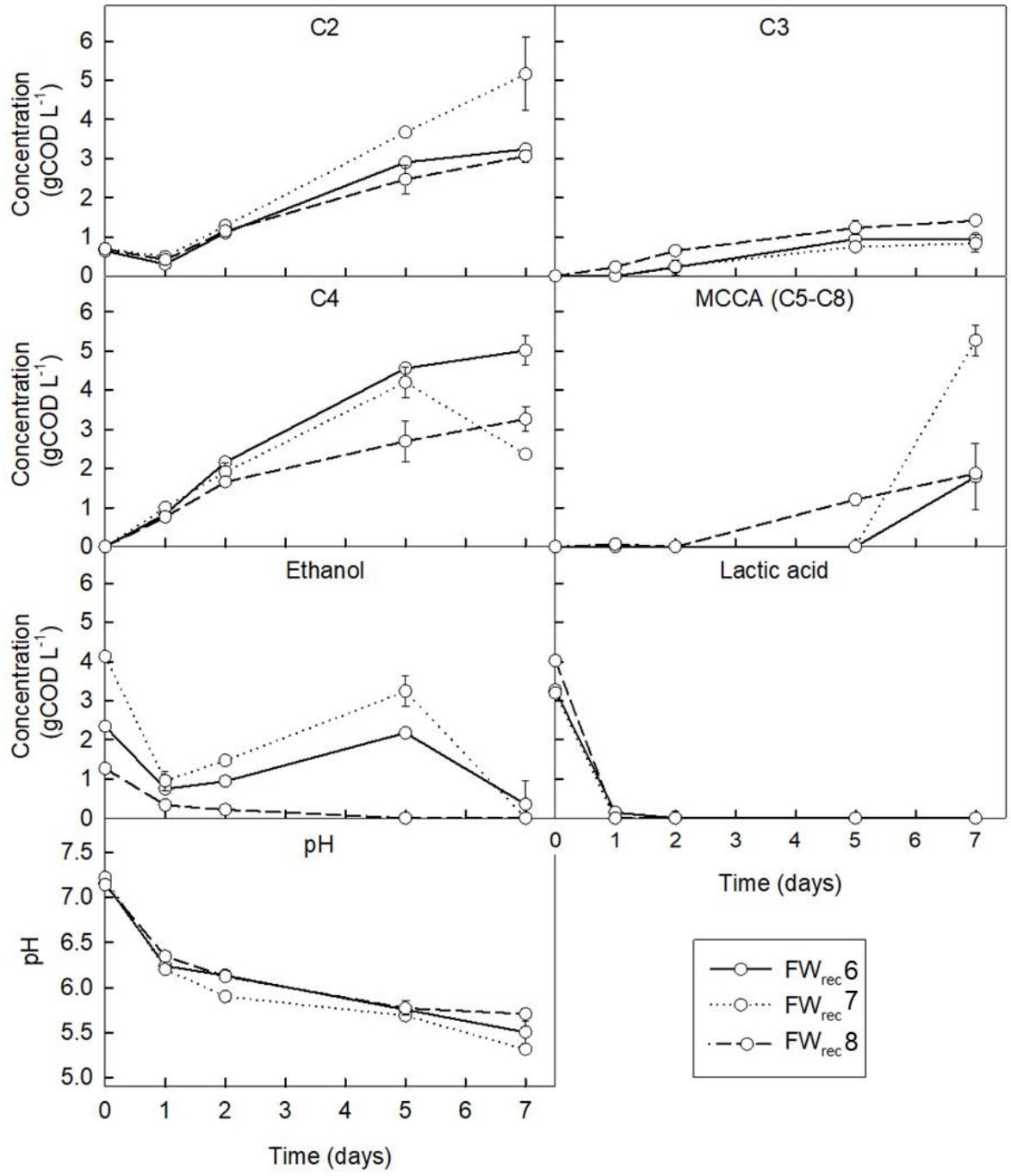


Figure 6-4 *Average concentration and pH profiles of liquid fermentation products in batch fermentation of food waste collected at a recycling centre (FW_{rec}) at 3 different times (numbered according to collection in Table 6-1). Error bars present standard deviations over triplicate reactors.*

Table 6-6 *Performance parameters for the acidogenic fermentation potential tests of the same feedstock collected at different times. Feedstocks tested were a mixture of cafeteria food waste and soybean processing wastewater (FW_{caf}:Ysoy where superscripts [a] and [b] indicate collection at different time) or food waste from a recycling centre (FW_{rec} numbered according to collection in Table 6-1).*

	FW_{caf}^{a}:$YSoy^{a}$	**$FW_{caf}^{b}$$YSoy^{b}$**	**FW_{rec} 6**	**FW_{rec} 7**	**FW_{rec} 8**
F/M	5	5	5.6 ± 0.1	5.6 ± 0.3	4.8 ± 0.1
Final pH	3.69 ± 0.00	3.94 ± 0.02	5.5 ± 0.1	5.31 ± 0.04	5.71 ± 0.04
Net Y_{VFA} (C1-C4) (%)	6.5 ± 0.4	21.9 ± 0.5	34 ± 2	31 ± 5	34 ± 3
Net Y_{MCCA} (C5-C6) (%)	0.3 ± 0.3	0.1 ± 0.1	6 ± 3	21 ± 2	9.1 ± 0.2
Net $Y_{lactate}$ (%)	40 ± 1	18.3 ± 0.4	-13 ± 0	-13 ± 0	-20 ± 0
Net $Y_{ethanol}$ (%)	27 ± 2	10.2 ± 0.2	-8 ± 2	-17	-6 ± 0
CH_4 in biogas* (%)	7 ± 3	18 ± 3	18 ± 4	23 ± 5	30 ± 3
TS removal (%)	32.1 ± 0.2	35 ± 3	53 ± 12	35 ± 5	42 ± 7
VS removal (%)	34.4 ± 0.5	37 ± 5	62 ± 14	32 ± 3	53 ± 11

*Averaged over each sampling day

6.3.4. Increased sucrose in food waste stimulates butyric acid production in batch

6.3.4.1. Higher quantities of available sugars do not enhance MCCA production

MCCA production in semi-continuous FW fermentation is improved when using a high-COD feedstock (Chapter 3)[23]. MCCA yields were also higher in batch tests with a fresh feedstock, FW_{caf}, compared to FW_{rec} if pH was corrected, due to a greater content of biodegradable matter (Section 6.3.3.2.). It was hypothesised that a feedstock with more readily degradable organics stimulates chain elongation by providing more *in situ* produced lactic acid. Thus, AFP tests were performed with FW_{rec} supplemented with different proportions of sucrose, but with the same organic load (25 gCOD L^{-1} substrate for 5gVS L^{-1} inoculum) (Figure 6-5). Sucrose was supplemented as it is the most abundant sugar in many food products, such as fruits and grain legumes, and lactic acid bacteria have a range of metabolic pathways to consume it [55]. Additionally, some chain elongation bacteria consume sucrose, glucose or fructose for C6 production [56-58].

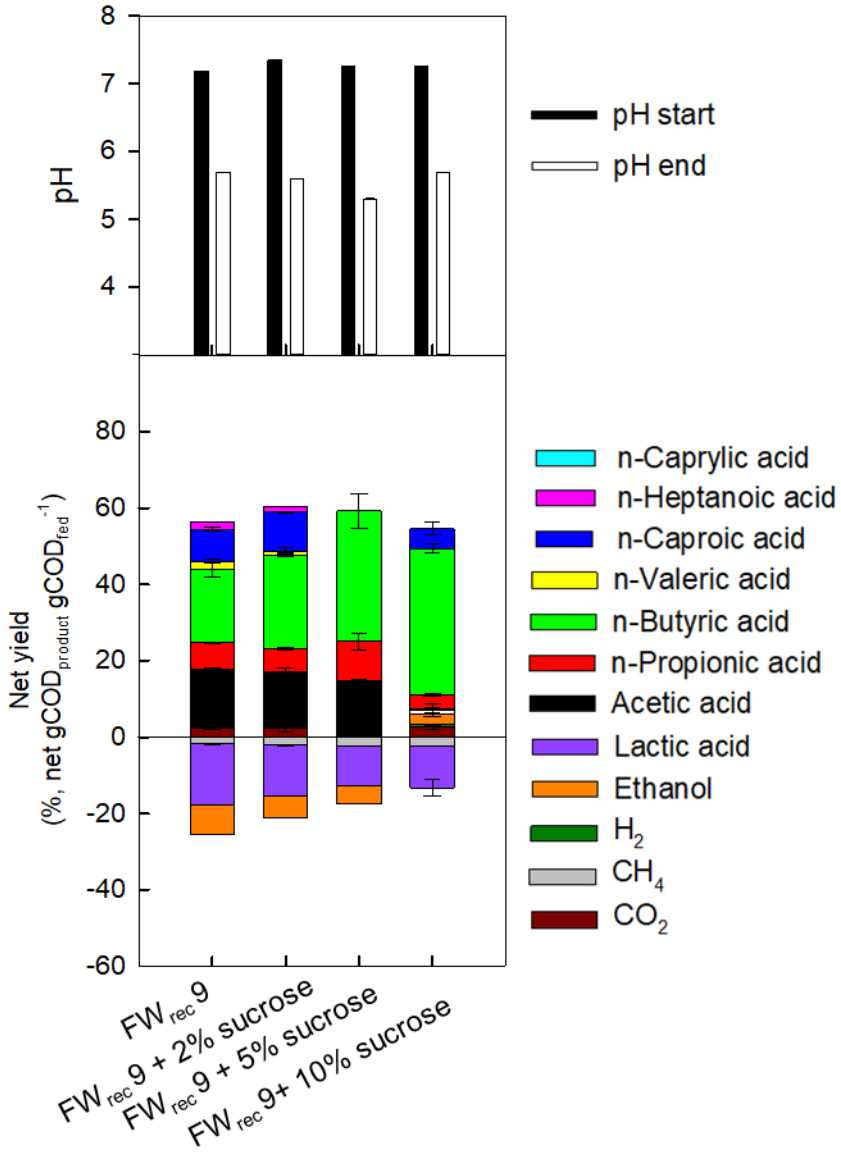


Figure 6-5 *The pH profile (top) and net product yields (bottom) of the AFP tests evaluating the acidogenic fermentation of food waste collected from a full-scale AD plant (FW_{rec} 9 in Table 6-1) with different amounts of enrichment of sucrose (w/w%). Negative yields for biogas compounds indicate less gas was produced than in the accompanying blank assay. Negative yields for liquid compounds indicate they were net consumed from the feedstock.*

Sucrose addition did not improve C6 yields (Figure 6-5). Adding 2% w/w sucrose gave a similar product spectrum to fermentation of pure FW_{rec}, predominantly carboxylates, and slightly increased the overall conversion to liquid products, including chain elongation. A different product spectrum occurred when 5 or 10% w/w sucrose was added. The yields of C4 increased with 5% w/w sucrose addition and at 10% w/w addition (38 ± 1%) were nearly double those for fermentation of pure FW_{rec} (19 ± 2%). H_2 was only detected (0.25 ± 0.04 L L^{-1}) at 10% w/w sucrose addition, ethanol was produced, and lactic acid not fully consumed. Thus, primary fermentation increased with addition of 5 or 10% sucrose but chain elongation bacteria did not take advantage of the increased available substrate.

6.3.4.2. Sucrose increased intermediate lactic acid and acidification

Previous tests showed that a small addition of sucrose (+2% w/w) improved carboxylic acids production. A 7-day AFP test with intermittent sampling was performed on two different FW_{rec} samples (to account for inherent variations), enriched with 2% w/w sucrose, to evaluate the fermentation pathways. (Table 6-7). The pH decreased after 1 day of fermentation, with a minimum of 5.42 ± 0.06 for FW_{rec} 7 + 2% sucrose. To avoid acid inhibition, NaOH was dosed to increase pH to 5.67 ± 0.03.

Table 6-7 *Performance parameters for the acidogenic fermentation potential tests with intermediate sampling of food waste collected at a recycling centre (FW_{rec}) at different times (numbered according to collection in Table 6-1) and enriched with 2% w/w sucrose.*

	FW_{rec} 7	**FW_{rec} 7 + 2% sucrose**	**FW_{rec} 8**	**FW_{rec} 8 + 2% sucrose**
F/M	5.6 ± 0.1	5.6 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
Final pH	5.31 ± 0.04	5.14 ± 0.08[$]	5.65 ± 0.02	5.64 ± 0.03
Net Y_{VFA} (C1-C4) (%)	31 ± 5	42 ± 4	38 ± 2	40.3 ± 0.7
Net Y_{MCCA} (C5-C6) (%)	21 ± 2	1.5 ± 0.1	9.4 ± 0.8	9.6 ± 0.4
Net $Y_{lactate}$ (%)	-13.0 ± 0.0	- 11.5 ± 0.0	-18.0 ± 0.0	-5.4 ± 0.0
Net $Y_{ethanol}$ (%)	-16.5 ± 0.0	- 9.3 ± 0.7	- 6.5 ± 0.0	-15.6 ± 0.0
CH_4 in biogas* (%)	23 ± 5	1 ± 2	8 ± 2	4 ± 2
TS removal (%)	35 ± 5	33 ± 4	30 ± 3	27 ±
VS removal (%)	32 ± 3	52 ± 9	37 ± 5	37 ± 6

*averaged over each sampling day; [$]NaOH dosed on sampling days if pH <5.5

Sucrose stimulated *in situ* lactic acid production as almost no lactic acid was detected after 24 hours of fermentation of FW_{rec}, whereas it was present with sucrose supplementation (Figure 6-6). Lactic acid is generally the main fermentation product when easily degradable sugars are available, due to the higher specific biomass uptake rate for sugars of lactic acid bacteria (LAB) compared to other fermentative bacteria [48, 59]. The final product was predominantly C4 for all feedstocks. It is likely that the lactic acid produced after Day 1 was converted to C4, as C4 concentrations peaked after consumption of lactic acid. Concentrations of C4 were consistently higher with sucrose addition than the un-supplemented test. Chain elongation of C4 to C6 occurred via ethanol as FW_{rec} 7 and FW_{rec} 8 showed inversely proportional rates of ethanol consumption and C6 synthesis. Addition of sucrose either did not affect chain elongation (FW_{rec} 8 + 2% sucrose) or affected it negatively, potentially by lower pH, as more intermediate ethanol and less C6 was found for FW_{rec} 7 + 2% sucrose. Lactic acid was thus elongated to C4, but not to C6. Certain bacteria in the genus of *Clostridium* lead to lower MCCA yields during long-term experiments as they elongate lactic acid to C4 with acetic acid but do not elongate further

to C6 [60-62]. The additional sucrose increased *in situ* lactic acid synthesis and subsequent fermentation to C4, but chain elongation to C6 occurred predominantly via ethanol, which was mainly unaffected or potentially slowed down by a lower pH.

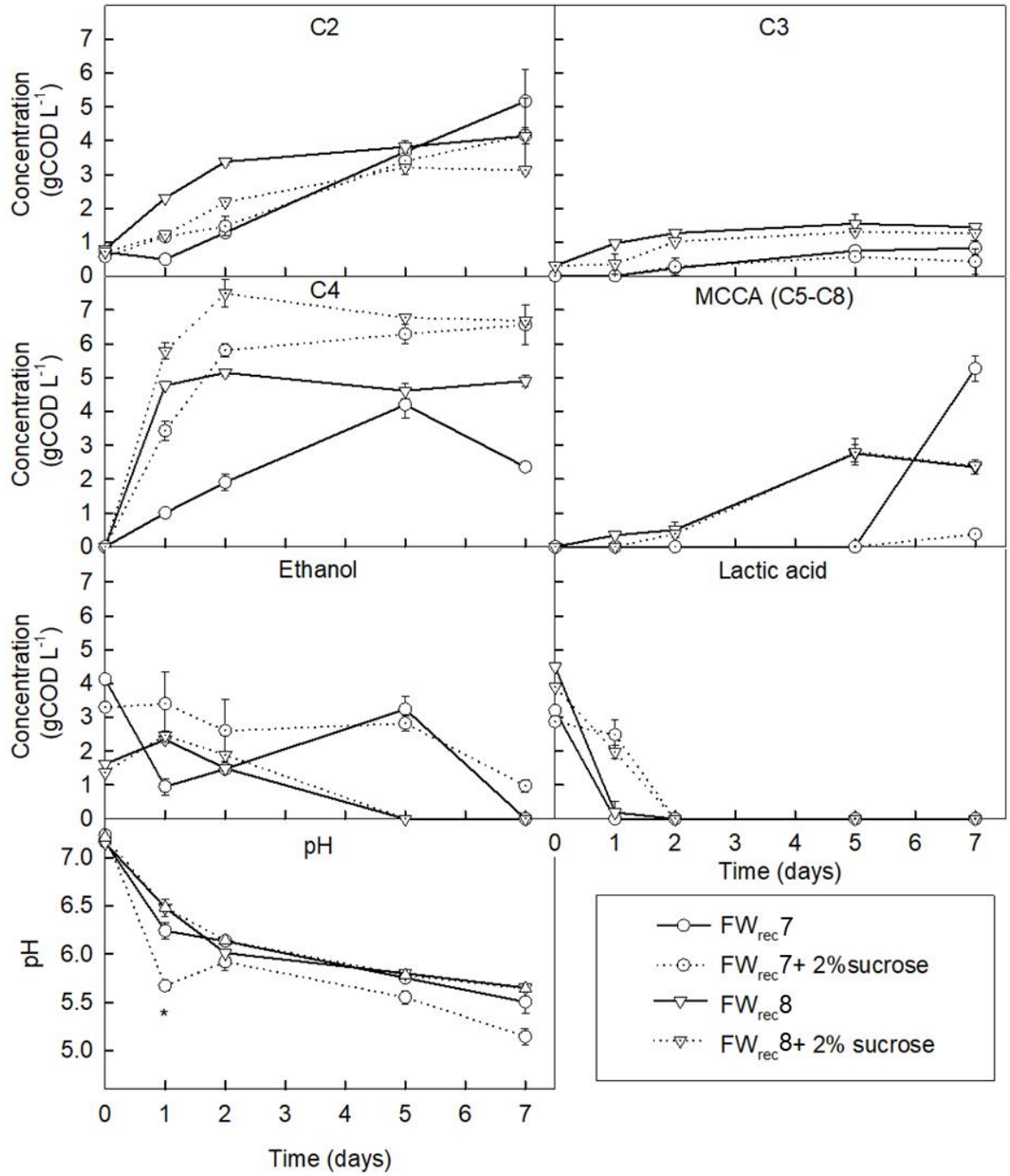


Figure 6-6 *Average concentration and pH profiles of liquid fermentation products in batch fermentation of food waste collected at a recycling centre (FW_{rec}) at different times (numbered according to Table 6-1) and enriched with 2% w/w sucrose. Error bars present standard deviations over triplicate reactors. *··○·· NaOH was added to increase pH in the reactors from 5.42 ± 0.06 to above 5.5 to avoid acid inhibition.*

6.3.5. Long-term effects of easily biodegradable sugars in FW

To determine how increased availability of sucrose affects the microbial community, a long-term reactor experiment was performed. Two STRs were operated semi-continuously in duplicate, in a similar manner to long-term reactor operation in Chapters 3 and 4, with a FW feedstock supplemented with 2% w/w sucrose. In this case pH was corrected every 3.5 days with NaOH after introducing new feedstock, thus decreasing exposure to acidic pH.

6.3.5.1. Sucrose in food waste destabilises fermentation with lactic acid accumulation

During the first 60 days of operation (4 HRT), a mixed acid type fermentation was obtained, as characterised by consumption of lactic acid from the feedstock and C4 and C6 production (Figure 6-7). Several peaks of net lactic acid production occurred in both reactor replicates (Days 38.5 and 49 for Reactor 1, and Days 17, 38.5 and 56 for Reactor 2, Figure 6-7). During the following 30 days lactic acid was the predominant product, reaching concentrations up to 50 g COD L^{-1}. Near the end of reactor operation lactic acid dropped and C4 and C6 were produced again. Thus, the presence of lactic acid in the reactor appeared to be inversely proportional to carboxylic acid production, as found with co-fermentation of FW_{caf} and Ysoy under analogous operation (Chapter 5).

Correlation analysis confirmed lactic acid yield was negatively correlated with the yields of C4, C5 and C6 ($p<0.05$, Figure 6-8), indicating chain elongation via consumption of lactic acid. Lactic acid yield was positively related to C2 yield and acidification, i.e., the amount of NaOH required to bring pH to 5.5 at point of feed addition. Both C2 and lactic acid are products from primary fermentation and lead to a decrease in pH. Thus, the lactic acid was switching from final product to intermediate substrate.

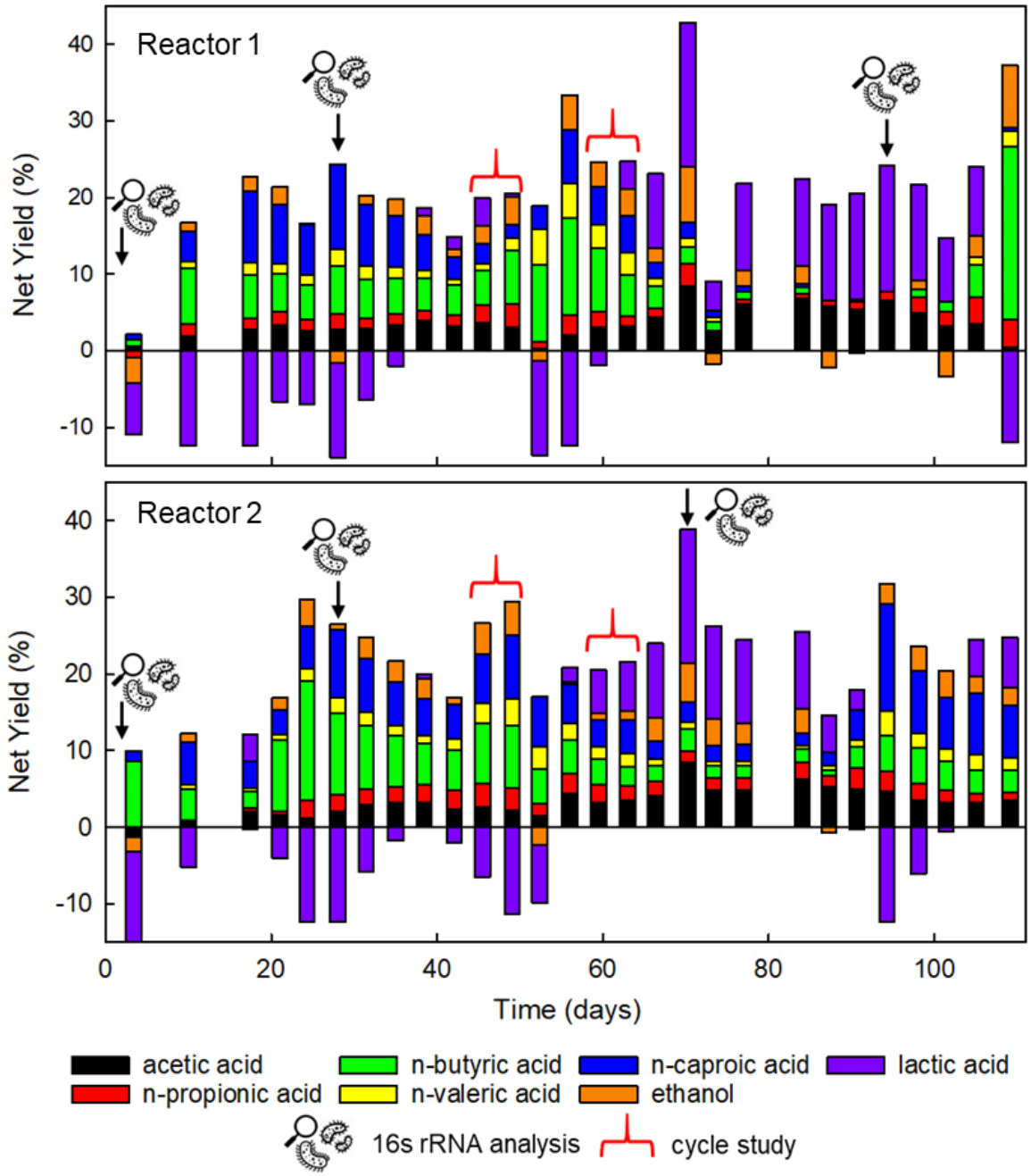
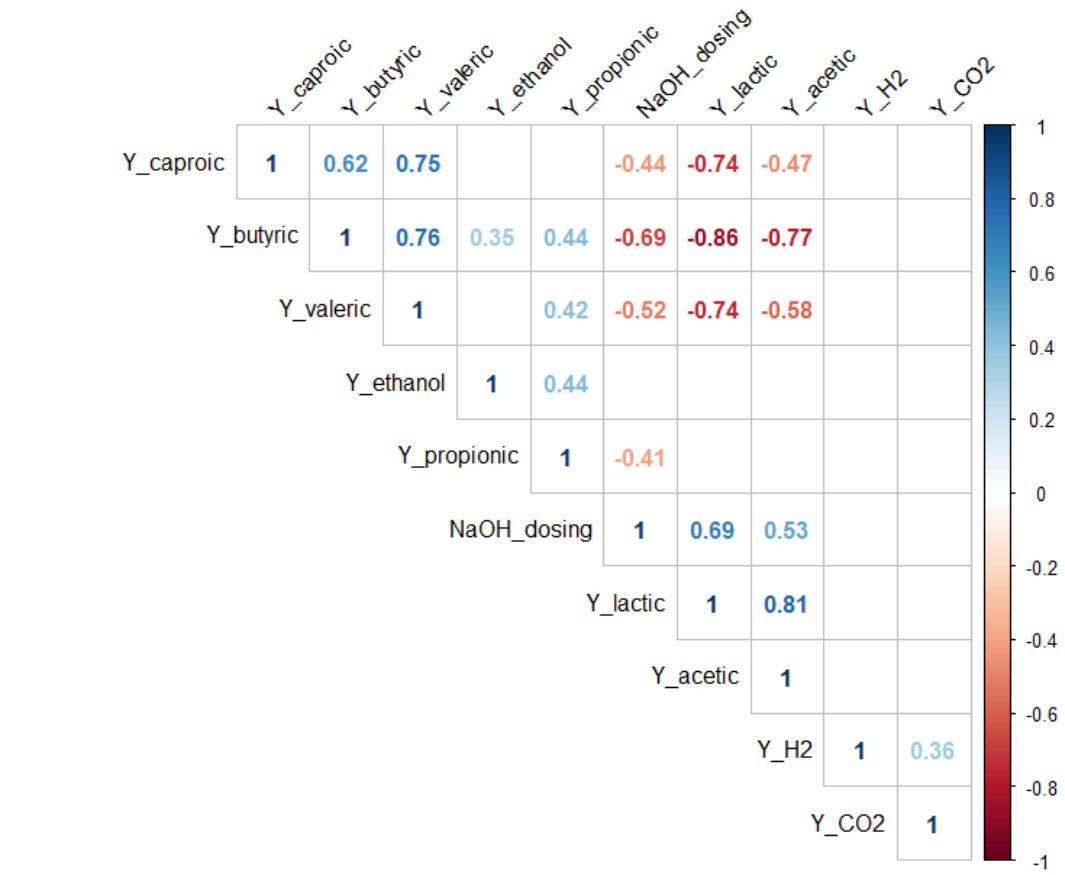


Figure 6-7 *The liquid product yield from fermenting food waste enriched in 2% w/w sucrose in duplicate. Negative values indicate net consumption, positive indicate net production. Arrows indicate where biomass samples were taken for microbial community analysis (16s rRNA sequencing). Red brackets indicate points where cycle studies were performed, i.e., fermentation compounds were analysed in between two feeding events.*

A.



B.

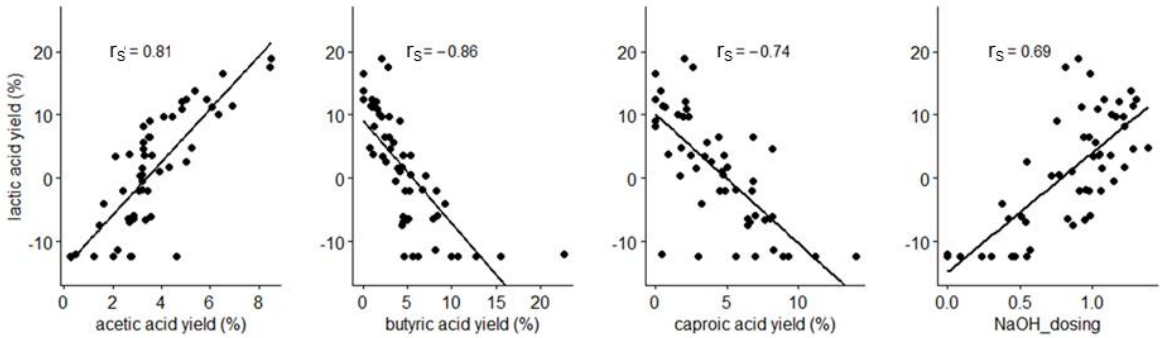


Figure 6-8 *Correlation analysis of product yields and acidification (NaOH_dosing) in the two reactors. (**A**) Spearman correlation matrix where the variables on the left of the graph are significantly correlated ($p<0.05$) with the variables on the top by the correlation coefficient (r_S) given in the overlapping squares. The closer the r_S is to 1 or -1, the more the two variables are positively or negatively correlated. (**B**) The scatter plots present the correlation ($p<0.05$) between the data points of lactic acid yield and, from left to right, acetic, butyric and caproic acid yield and NaOH dosing. The amount of NaOH required to correct for minimum pH at feeding events (NaOH dosing, g $L^{-1}d^{-1}$) is representative of the acidification during fermentation. Data points from < 1 HRT operation are omitted, n = 52.*

6.3.5.2. Presence of chain elongation precursors does not necessarily lead to chain elongation

The fermentation cycles were studied during a phase of chain elongation (Cycle Study 1, Days 45.5 to 49) and when primary fermentation to lactic acid prevailed (Cycle Study 2, Days 59.5 to 63). In all cases the pH decreased during the first 24 hours after feed addition via primary lactic acid fermentation. In Cycle Study 1 where net chain elongation took place, the pH increased again due to net consumption of lactic acid and production of C4, C6 and H_2 (Figure 6-9 A). This profile with consecutive fermentation stages is typical for single-stage FW fermentation [23, 38]. The kinetic AFP batch tests in Section 6.3.3.3. showed MCCA production followed a net consumption of ethanol. In these cycle studies on semi-continuous fermentation, lactic acid appeared to be used instead of ethanol in chain elongation. Conversion reactions in anaerobic communities are flexible as they occur close the thermodynamic equilibrium, and follow the most efficient catabolic systems due to syntrophic interactions [63]. These behaved differently when comparing batch FW fermentation with a non-acclimatised inoculum to semi-continuous operation with an enriched microbial community.

During Cycle Study 1, Reactor 1 showed more conversion of lactic acid to C4, whereas Reactor 2 gave more C6, H_2 and CO_2 production, which is typical for chain elongation of lactic acid to MCCA. In Reactor 1, the pH dropped to 5.12 compared to Reactor 2 which stabilised around 5.5. It could be that elongation to C6 was hindered in Reactor 1 by the lack of H_2 accumulation, as less H_2 was produced compared to Reactor 2, or that pH became too low. The latter is unlikely since the pH in Reactor 2 increased from 5.12 to 5.32 after primary fermentation, indicating a second fermentation phase, and chain elongation to C6 can still occur down to pH 5.0 [60]. The role of H_2 in these systems is still unclear as it is both a consequence of and a driver for microbial chain elongation. On one hand it is a product from ethanol oxidation, which precedes the reverse β-oxidation pathway to make chain elongation energetically feasible, and on the other hand its presence is critical to ensure sufficiently reduced conditions for microbial chain elongation [64]. Experimental trials have confirmed that a supply of H_2 improved lactate-based chain elongation to C6 [65]. In organic waste fermentation with mixed microbial communities, H_2 can accumulate via various fermentation pathways, such as carbohydrate degradation [66, 67]. Further analysis of the interplay between the metabolic pathways resulting in H_2 or chain elongation when fermenting complex organic waste such as FW should allow improved process design.

In Cycle Study 2, chain elongation did not follow primary lactic acid fermentation even though all necessary precursors, i.e., ethanol, lactic acid and VFA, were present. In Cycle Study 2, less biogas was produced (H_2 and CO_2) and fewer changes in carboxylic acid content were observed (Figure 6-9 B), indicating reduced fermentation activity overall. The lack of available H_2, or acid inhibition, could explain the lack of chain elongation.

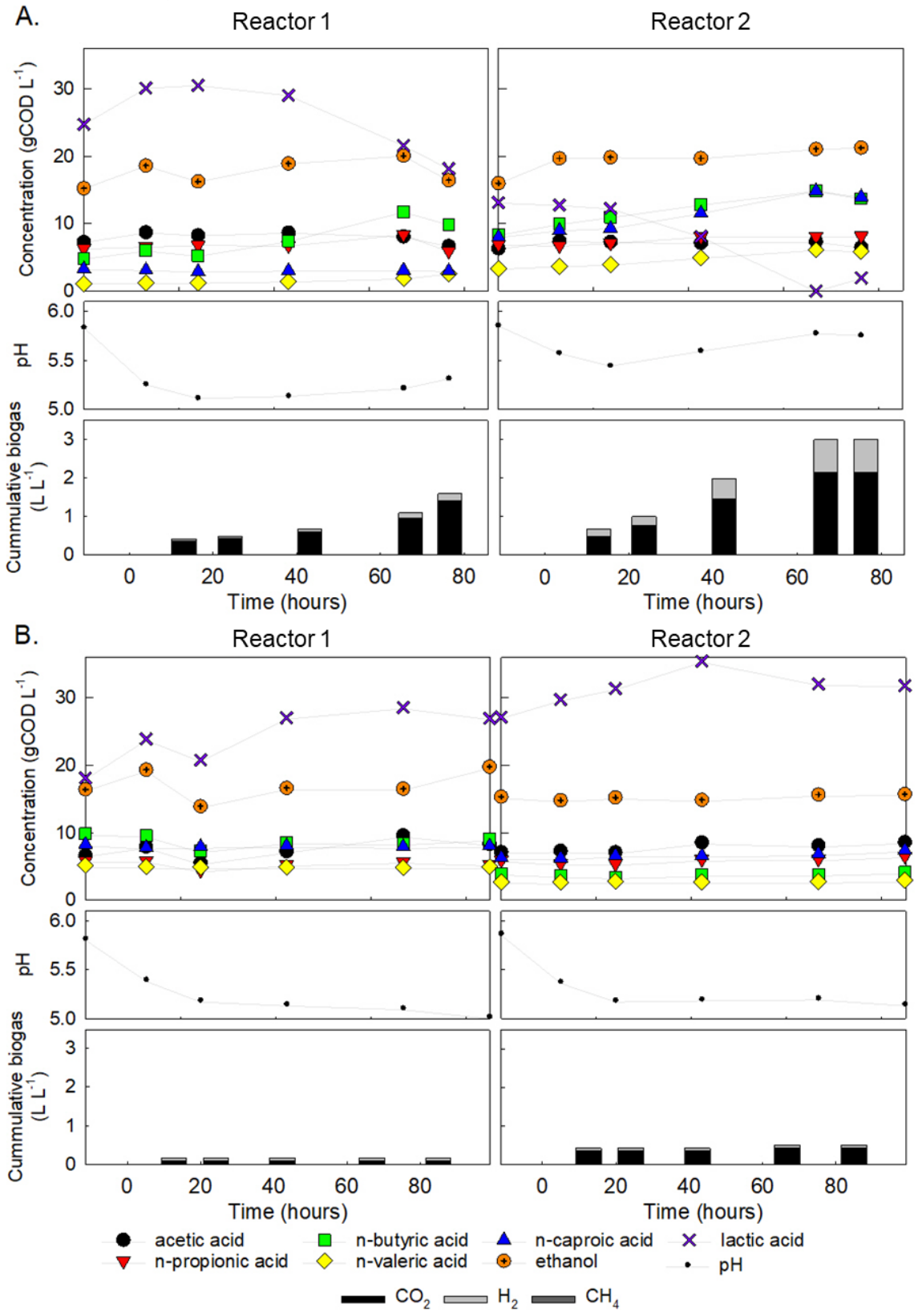


Figure 6-9 *Comprehensive overview of cycle studies performed in duplicate over (A) Day 45.5 to 49 with net chain elongation and (B) Day 59.5 to 63 with net lactic acid production. Data is included from both reactors (left vs. right) regarding the liquid product concentration (top) and pH (middle) measured in the fermentation broth from point of feed addition (time = 0 hours), and cumulative biogas production as measured from the gas collection tubes (bottom).*

6.3.5.3. Microbial community reflects fermentation outcome

To see whether the fluctuations in fermentation outcome correlated with the microbial community composition, duplicate biomass samples were analysed for each reactor from the inoculum on Day 0, during a C6 concentration peak (Day 28), and during lactic acid accumulation (Day 94 for Reactor 1 and Day 70 for Reactor 2). All samples showed a low alpha-diversity due to some highly dominant genera belonging predominantly to the phyla of Firmicutes, Bacteroidetes and Actinobacteria (Figure 6-10). The effective alpha-diversity numbers, ^{1}D and ^{2}D, and dominant genera in the microbial communities were similar to those performing acidogenic fermentation with FW_{rec} or co-fermentation of FW_{caf} and Ysoy (Chapters 3-5).

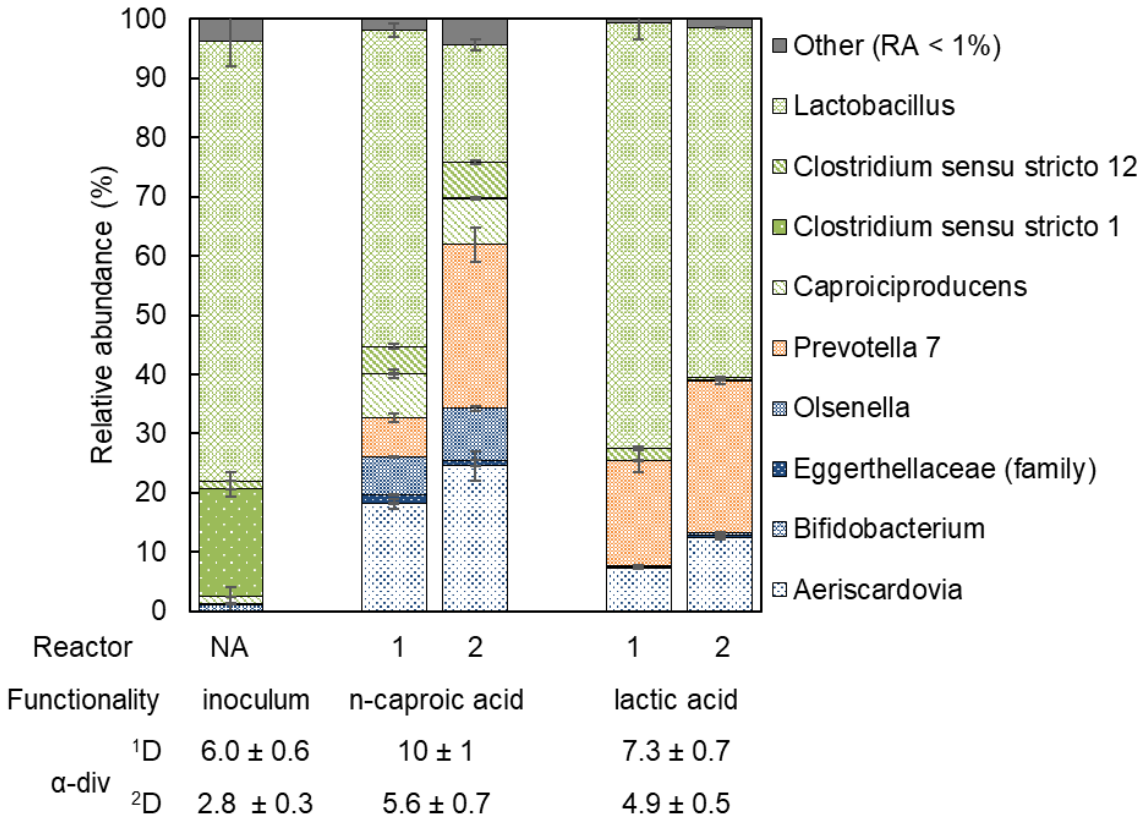


Figure 6-10 *Microbial community composition on the genus level (unless specified otherwise) with a relative abundance >1% and effective alpha-diversity indices (1st and 2nd order Hill number, ^{1}D, ^{2}D). Communities are compared in the inoculum (n=4, 2 reactor duplicates and 2 sample duplicates), and in each reactor (1 or 2, n=2 samples per reactor) sampled at a peak in n-caproic acid concentration (Day 28) and lactic acid accumulation (Day 94 for Reactor 1 and Day 70 for Reactor 2). Colours reflect phylum: green = Firmicutes; orange = Bacteroidetes; blue = Actinobacteria.*

The genus with the highest relative abundance was *Lactobacillus* spp. at nearly double in the lactic acid accumulation phase (65 ± 8%), compared to the C6 concentration peak (37 ± 20%); this agrees with the predominant metabolism of this genus. Other abundant genera were *Aeriscardovia* spp. and *Prevotella 7* spp., which are typical of acidogenic FW

fermentation where the former produces lactic acid, C2 and ethanol, and the latter converts lactic acid to VFA such as C4 [68, 69]. In the samples at high C6 concentrations, the C6-producing *Caproiciproducens* spp. (7.6 ± 0.5%) and the lactic acid producing *Olsenella* spp. (8 ± 1%) were detected, whereas their relative abundance was less than 1% during the lactic acid accumulation phase. *Clostridium senso stricto 12* spp., reported to produce C4 and compete with chain elongation for C6 production [61], were also more abundant in the C6 phase (5.3 ± 0.9 %) than the lactic acid phase (1.3 ± 0.9 %). Thus, whilst the most abundant OTUs were similar for all reactor samples, the microbial community composition did change according to reactor phenotype observed. This is consistent with results from co-fermentation of fresh FW feedstock, FW_{caf} and Ysoy (Chapter 5).

Within the genus of *Lactobacillus* spp.*,* 31 different OTUs were identified, with certain OTUs having a far higher relative abundance than others depending on the dominant fermentation product. For instance, OTU_6, classified in the genus of *Lactobacillus*, had a higher relative abundance during the lactic acid phase (25 ± 3%) than during the C6 phase (4 ± 2%). This OTU was previously described in Chapter 4 and has a homolactic metabolism. It was more abundant in lactate-producing reactors operated at an HRT of 8.5 days and an OLR of 20 gCOD $L^{-1}d^{-1}$, compared to the C6-producing reactors operated at lower OLR (12 gCOD $L^{-1}d^{-1}$) and higher HRT (10.5 days) (Chapter 4)[24]. The current reactors fed with FW_{rec} + 2% w/w sucrose were operated at a similar OLR of 11 gCOD $L^{-1}d^{-1}$ and an even longer retention time of 14 days to stimulate chain elongation, yet instability related to acidogenic lactic acid production occurred. The addition of sucrose to FW_{rec} enriched similar OTUs responsible for homolactic fermentation that were also observed when operating at higher OLR with pure FW_{rec}. The sucrose content in the FW feedstock, and the generally larger amount of easily biodegradable carbohydrates, will affect the optimal OLR/HRT combination for production of MCCA or other fermentation products.

In Chapter 5, the fluctuations between MCCA and lactic acid production were linked to potential changes in the feedstock, which was freshly prepared twice a week (FW_{caf} blended with soybean wastewater). However, here the operating conditions and substrate were the same, providing no potential reason for this unstable performance in the fermentation of FW_{rec} + 2% w/w sucrose. The drivers of microbial community evolution are still being investigated in the field of ecological theory due to the many factors that can influence it [70]. Improved understanding of these factors would allow more strategic design of reactor operating conditions to minimize competitive interactions and mitigate the risk of reactor failure [71]. An example of this could be adjusting OLR depending on the sugar content in the feedstock.

6.3.6. Natural phase separation of MCCA

During the fermentation of FW_{caf}:Ysoy (Chapter 5) and for fermentation of FW_{rec} supplemented with 2% w/w sucrose (Section 6.3.5.) reactors occasionally contained an immiscible low-density layer that separated from the bulk broth if left to sit. The top layer was a clear, reddish oil in the reactors processing FW_{caf}:Ysoy, whereas, it looked more greasy and had a slurry-like consistency in the reactors fed with FW_{rec} + 2% sucrose (Figure 6-11). The top layer was separated and analysed each reactor system.

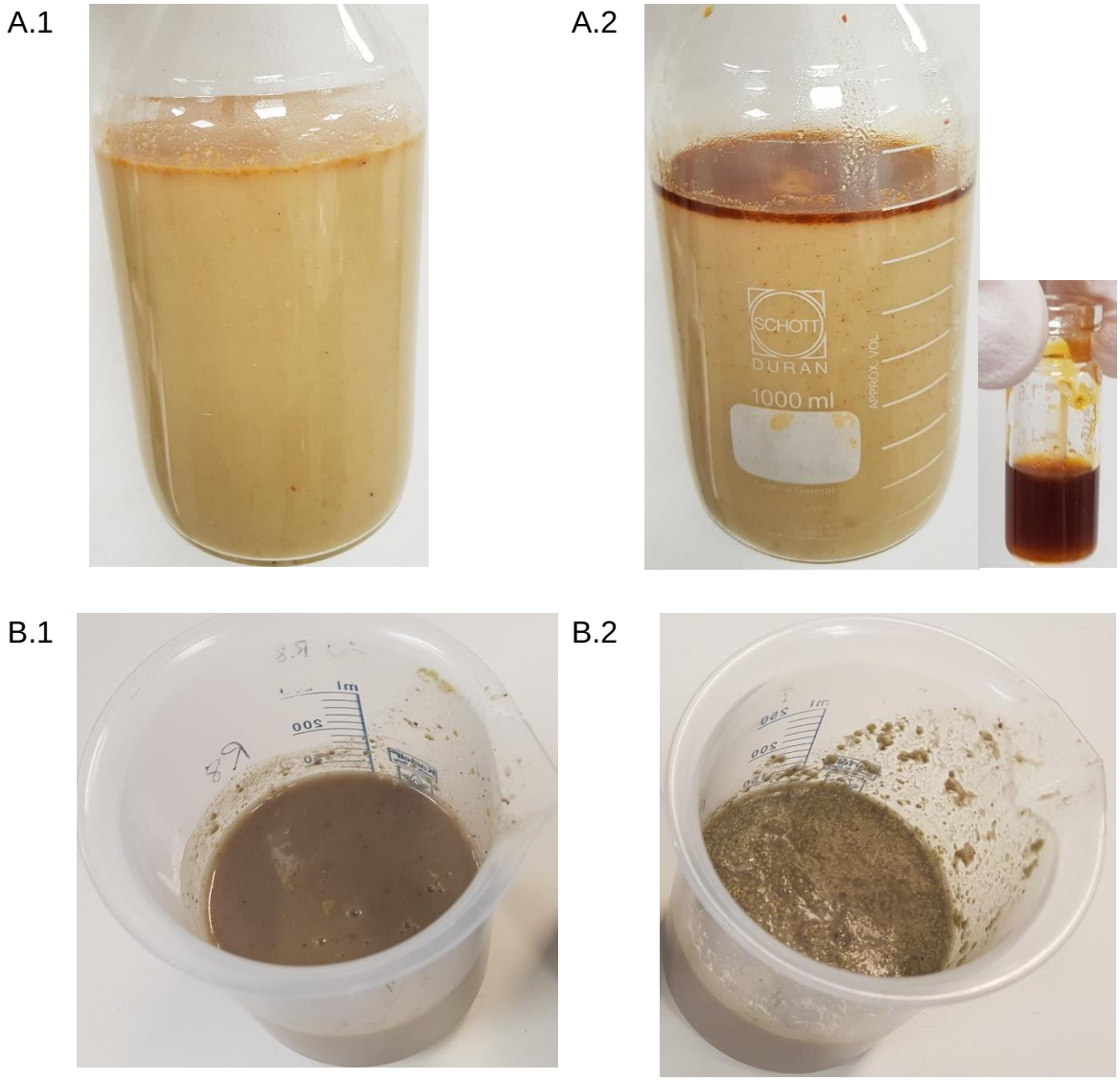


Figure 6-11 *Examples of reactor effluent where phase separation was observed. (A) effluent from fermentation of FW_{caf}:Ysoy Day 76.5 (Chapter 5). (B) effluent from fermentation of FW_{rec} with 2% w/w sucrose was fermented on Day 90 (Chapter 6, Section 6.3.5.). A.1 and B.1 show effluent from the reactor at that time that showed minimal phase separation. A.2 and B.2 show effluent from the other reactor operated in duplicate where an immiscible lower-density layer was observed and separated.*

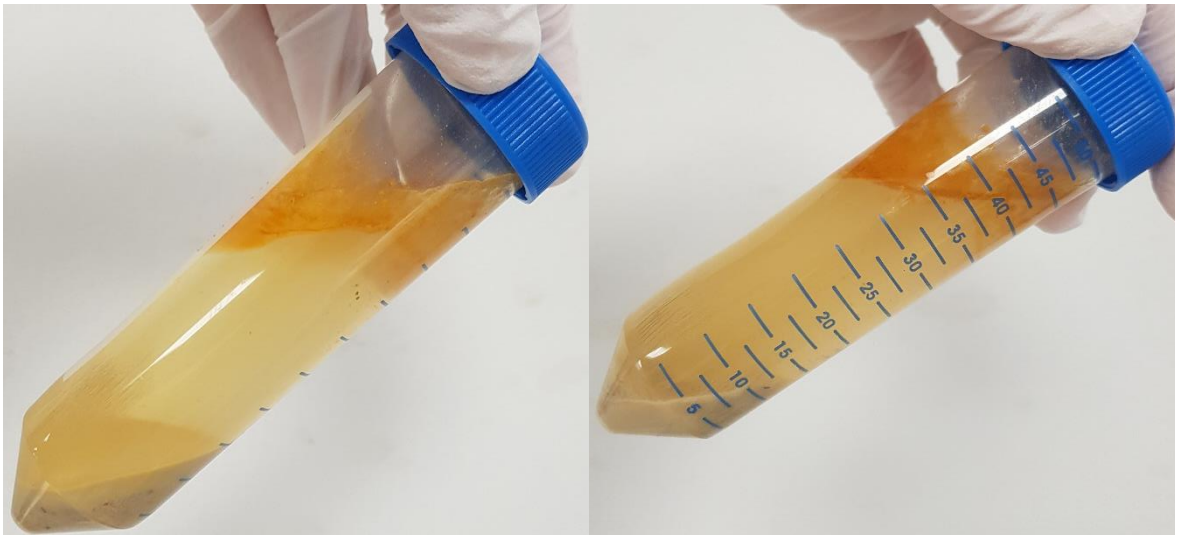
6.3.6.1. Cafeteria food waste fermentation results in a C8-rich top layer

The immiscible top layer seen in the effluent in Figure 6-11 A.2 was separated from the broth by pipetting, and the MCCA concentrations were measured and compared to the bulk liquid. C6 and C8 were enriched 1.7 and 22 times in the top layer, respectively, compared to the bulk concentration (Table 6-8). C6 and C8 have low solubility in water, and a lower density than water, so it is feasible that they would separate from the aqueous fermentation broth and become enriched in a non-aqueous phase. C8 is more hydrophobic than C6 and was therefore more concentrated in the oily layer.

The feedstock only contained an oily top layer after centrifugation (10min × 11000rpm, Figure 6-12). This was attributed to the oil-fraction in the cafeteria food from the cooking and seasoning oils. C6 and C8 naturally occur in pure plant oils such as coconut oil and palm kernel oil (0-10 w/w %)[72]. However, they were not detected in the feedstock. The presence of an oily component in the FW enhanced the natural extraction of hydrophobic product compounds from the aqueous fermentation medium.

Table 6-8 *Concentration of n-caproic (C6) and n-caprylic (C8) acid in the top layer and the bulk liquid in reactor effluent (collected on Day 76.5, a bi-weekly fed semi-continuous reactor processing cafeteria food waste and soybean soaking wastewater, pH = 6.01) and their maximum solubility in water at 20 °C [73].*

		C6	C8
Top layer (analytical duplicate)	**(g L^{-1})**	19.1 ± 0.4	79 ± 3
Bulk phase	**(g L^{-1})**	11.3	3.6
Maximum solubility in water	**(g L^{-1})**	10.8	0.68
	(gCOD L^{-1})	23.8	1.7



A. Feedstock B. Effluent

Figure 6-12 *Example of the three different layers observed after centrifuging (10min × 11000rpm) the feedstock (blend of cafeteria food waste and soybean soaking wastewater) or the reactor effluent. The solid layer settled to the bottom with a middle aqueous phase and an orange, oily top layer.*

6.3.6.2. Hydrophobic compounds separate into a fat layer

A floating greasy layer occasionally appeared in the reactors fed with FW_{rec} 7 and 2% sucrose (Section 6.3.5.) (Figure 6-11 B). After centrifugation (15min × 4,500rpm) of the reactor contents three phases could be distinguished: a layer of solids (Phase C), an aqueous supernatant (Phase B), and an insoluble fatty layer (Phase A) (Figure 6-13).

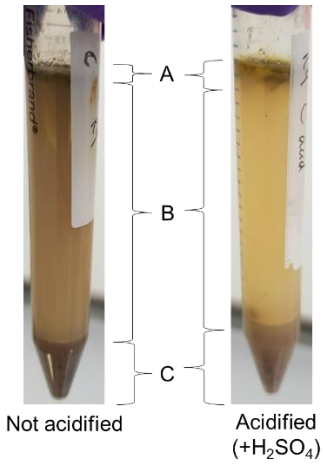


Figure 6-13 *Three phases could be distinguished after centrifugation of a reactor sample (15min × 4,500rpm). These phases were more distinct when samples were acidified by addition of 0.5mL of 50 % H_2SO_4 prior to centrifugation (right).*

The carboxylic acid content of the three different layers were compared to the concentrations in the bulk medium for three different reactor samples. A mass balance is available in Table 6-9, and results are visualised in Figure 6-14. One of the samples was acidified prior to centrifugation by addition of 0.5 mL of 50% H_2SO_4, since at lower pH the hydrophobicity of carboxylic acids increases due to the acid-dissociation equilibrium. At a lower pH, a larger fraction of carboxylic acids are protonated, and are hence less water soluble than their conjugate base or carboxylate form. They therefore separate from the aqueous phase more easily. The pKa of the target VFA and MCCA lies between 4.8 and 4.9 [74]. The shorter chain carboxylic acids (C2-C5) had similar concentrations in the three phases and the bulk for samples that were not acidified (Figure 6-14). However, in the case of the acidified sample, the concentration in the top layer (Phase A) was 2 and 6 times higher for C4 and C5, respectively (Table 6-9). Indeed, the lower the pH and the longer the alkyl chain of a carboxylic acid, the more hydrophobic it is. This effect was even more pronounced for C6. In the samples at near neutral pH (pH 6.68, Figure 6-14 Sample2), the distribution of C6 was similar for all 3 phases and the bulk. The total C6 concentration (22.04 gCOD L^{-1}) was just below the maximum water solubility (23.8 gCOD L^{-1}, Table 6-8), thus, no spontaneous phase separation occurred. By contrast, at lower pH, either naturally from the reactor (Sample 1 at 5.60 and Sample 3 at 5.65) or when the reactor sample was acidified (Sample 2, pH 1.20), C6 was enriched in the top Phase A, and to a lesser extent

in the solid Phase C. The concentration of C6 in the middle Phase B was reduced. The C6 concentration in Phase A peaked for the acidified Sample 2 at 117.6 gCOD L^{-1}, i.e., over 5 times higher than the bulk fermentation medium concentration.

Table 6-9 Data overview of the concentrations of the different carboxylic acids measured in the different phases of the effluent including mass balance.

Total measurement: bulk concentration (gCOD kg^{-1})					
	C2	**C3**	**C4**	**C5**	**C6**
Sample 1	10.37	5.86	5.22	1.39	5.92
Sample 2	8.47	4.32	8.25	4.92	22.04
Sample 3	8.66	3.78	7.68	4.58	19.22
Sample 2 - acid	8.47	4.32	8.25	4.92	22.04
Phase separation: Mass fraction (g)					
	total	**Phase A**	**Phase B**	**Phase C**	
Sample 1	48.59	1.18	40.15	7.27	
Sample 2	13.12	0.05	10.32	2.72	
Sample 3	13.41	0.57	10.13	2.69	
Sample 2 - acid	13.75	0.34	10.83	2.79	
Phase separation: Phase A concentration (gCOD kg^{-1})					
	C2	**C3**	**C4**	**C5**	**C6**
Sample 1	9.62	4.26	3.65	1.04	13.76
Sample 2	6.79	2.86	4.52	2.53	20.39
Sample 3	4.90	0.00	4.73	4.80	42.66
Sample 2 - acid	6.00	0.00	11.78	15.41	117.60
Phase separation: Phase B concentration (gCOD kg^{-1})					
	C2	**C3**	**C4**	**C5**	**C6**
Sample 1	7.96	4.75	4.79	1.20	4.86
Sample 2	8.92	4.58	8.86	5.19	22.79
Sample 3	8.59	3.79	7.79	4.51	17.31
Sample 2 - acid	8.34	4.11	7.40	3.52	9.08
Phase separation: Phase C concentration (gCOD kg^{-1})					
	C2	**C3**	**C4**	**C5**	**C6**
Sample 1	9.48	5.39	4.60	0.92	6.65
Sample 2	8.03	3.79	6.46	3.69	20.57
Sample 3	7.17	3.47	6.94	4.70	23.27
Sample 2 - acid	7.20	3.86	8.47	7.27	41.90
Phase separation: mass balance (Equations 6-2 and 6-3)					
	C2	**C3**	**C4**	**C5**	**C6**
Sample 1	23%	19%	10%	19%	10%
Sample 2	3%	2%	1%	1%	1%
Sample 3	6%	6%	3%	1%	2%
Sample 2 - acid	3%	7%	5%	6%	17%

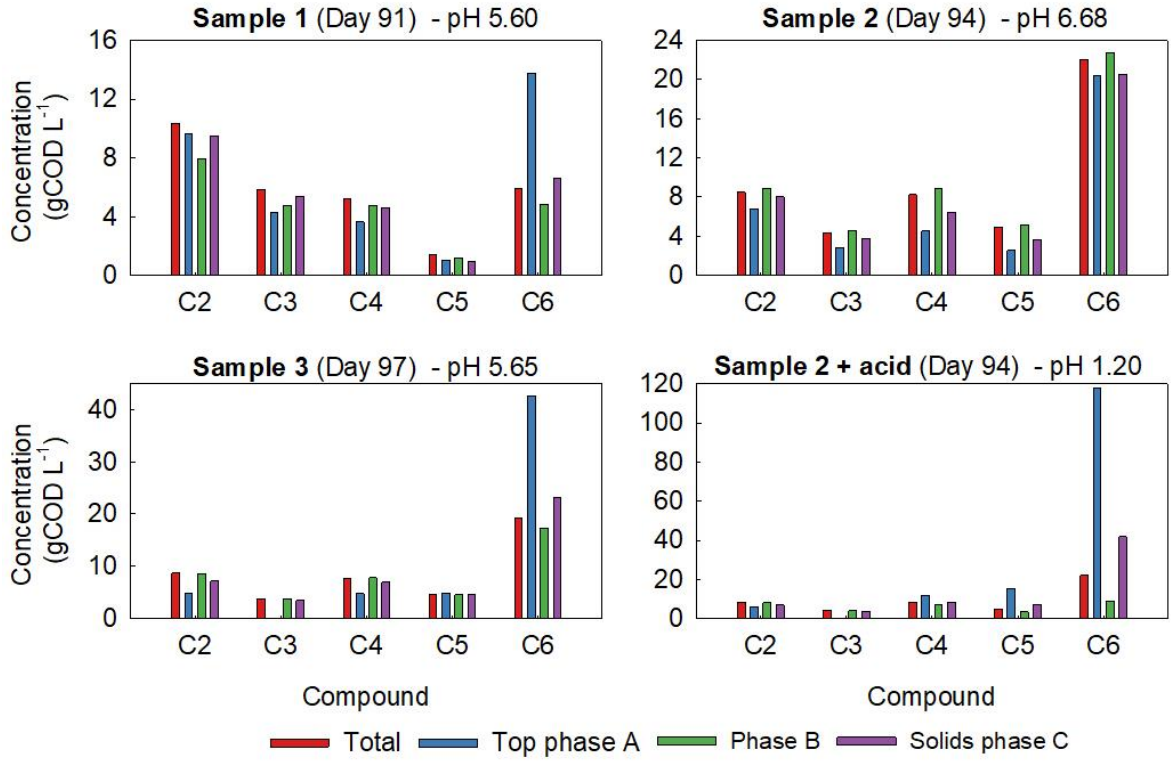


Figure 6-14 *The carboxylic acid concentration in the bulk liquid (total) and after centrifugation, in the immiscible top phase A, the middle liquid phase B, and the solid layer C for three effluent samples collected from Reactor 2 described in Section 6.3.5. with corresponding pH. Sample 2 was also analysed after acidification with H_2SO_4. Compounds analysed were acetic acid (C2), n-propionic acid (C3), n-butyric acid (C4), n-valeric acid (C5) and n-caproic acid (C6).*

6.3.6.3. Potential implications for spontaneous phase separation of MCCA

Oils and fats are expected in FW with average concentrations around 15.4 ± 8.0 %VS [1]. However these values can be higher, for instance 31.4 ± 0.04 %VS, depending on location [6]. The spontaneous separation of MCCA into an oil or solid phase is a positive benefit to commercial application, as it will simplify product recovery and downstream processing via the potential for *in situ* extraction. If FW feedstocks contained an oily component that is effectively inert during fermentation, this could be used to extract MCCA, and especially C8. *In situ* biphasic extraction of C6 from pure culture fermentation on sucrose has been demonstrated, but the synthetic extractants used had some toxic effects [75]. Using sunflower oil as diluent for the extractant reduced toxicity in the extraction of other carboxylic acids [76]. Further downstream processing would be possible using an oil-trap mechanism or a gravitational separation based on differential liquid density [77, 78]. Subsequent product purification could be performed as per traditional oleochemistry approaches [72].

Spontaneous removal of MCCA from the aqueous phase into an oily, fatty or solid phase is likely to improve chain elongation by reducing its presence (and hence toxicity/inhibition) within the aqueous phase, as the concentration that the microorganisms are exposed to

would be reduced. A similar concept was reported when MCCA was adsorbed onto biochar *in situ* [79]. This reaction/separation effect might have contributed to the high MCCA concentration reached in some of the FW fermentation studies presented in this thesis.

6.4. Conclusion

This chapter evaluated variations seen in the feedstock samples, which were within an expected range according to literature. However, this range is broad with, for instance, solid and COD content of solid cafeteria FW being triple those of slurry-like mixtures used in AD for FW recycling. For a FW feedstock collected from a recycling centre, it was observed that total COD can vary from 129 to 297 gCOD L^{-1} depending on time of collection. This is due to the range of liquid mixers used to turn the feedstock to a slurry suitable for pumping into the AD reactors, and variations in the feedstock itself. Batch tests indicated increased ethanol content stimulated MCCA production, whereas sucrose stimulated production of n-butyric acid. This can either be a challenge due to variability, or a potential opportunity to fine-tune the feedstock by careful blending. For instance, targeting lactic acid or C4 production by addition of sucrose-rich mixers, or targeting MCCA by using ethanol-rich or oily mixtures. Whilst improving targeted product generation, this could increase operation complexity.

This chapter highlighted how not all types of FW are the same when it comes to their use as feedstock for acidogenic fermentation. Low COD wastewaters are unsuitable by themselves, as they do not inhibit methanogenesis due to their low organic strength. However, they can be co-fermented with cafeteria FW to increase the organic content. Batch fermentation with cafeteria FW predominantly leads to an acidogenic lactic acid-type fermentation without pH control. By contrast, fermentation with FW from a recycling centre leads to accumulation of carboxylic acids, including MCCA. Different product outcomes are due to differences in readily biodegradable content resulting from variations in preparation and storage. Hence, storage should be optimised depending on the targeted fermentation product. Alternatively, pH control can be used to steer lactic acid type fermentation towards carboxylate accumulation, however this involves additional operational and environmental costs.

Interestingly, batch tests showed that chain elongation predominantly occurred via ethanol, whereas semi-continuous operation with an adapted microbial community leads to predominantly lactic acid-based chain elongation. Thus, both mechanisms may be exploited in acidogenic FW fermentation, and determining the drivers for each would allow the design of a system where both compounds are used optimally. For instance, in the semi-continuous

reactors, residual ethanol remained in the effluent. The production of H_2 could play an interesting role in this. Untangling the microbial pathways, and the interplay between H_2 fermentation from carbohydrates, and subsequent chain elongation, could reveal some interesting synergistic mechanisms which could be exploited to optimise resource recovery.

This chapter confirms the findings from Chapter 5, that the fermentation instability between lactic acid accumulation and carboxylic acid production results from a competition between homolactic *Lactobacillus* spp. and a team of primary fermenters and chain elongation bacteria. In Chapter 4 a lower OLR was shown to mitigate acidification and lactic acid accumulation, and a higher HRT promoted chain elongation. Hence, these operating conditions can be manipulated to direct these competitive processes one way or another. The level of biodegradable matter in the feedstock composition will determine the optimal values for OLR and HRT.

The amount of oils and fats in the feedstock influenced the process as well as the ethanol and sugar content. MCCA will spontaneously separate into an immiscible low-density layer at low pH due to their hydrophobic nature. This is unique for this type of acidogenic fermentation, namely chain elongation in mixed FW, and provides the potential for *in situ* product separation and hence simplified and less expensive MCCA recovery opportunities.

The suitability of acidogenic fermentation to produce MCCA from food waste as technology for resource recovery in recycling facilities requires a level of control of feedstock composition. Preparation, storage and pretreatment of the feedstock should be designed in a way that promotes chain elongation over other competitive processes such as homolactic fermentation or methanogenesis. Fine-tuning of the feedstock by blending different types of food waste is promising and could increase yields, and potentially facilitate downstream processing.

Acknowledgements

This chapter would not have been possible without the regular and reliable support and help in the collection of feedstock samples from Tom Phelps, Wesley Wong, and other technical colleagues from GENeco (Avonmouth, UK), Liew Chee Wai from SCELSE, and the staff at Quad Café (NTU, Singapore). In addition, experimental help was received from undergraduate MEng project students Emma Jarvis, Rosie Buzova, Chak Chan and Orla Douds (Department of Chemical Engineering, University of Bath, UK). I am also grateful for the experience in student supervision that this afforded.

References

[1] H. Fisgativa, A. Tremier, and P. Dabert, "Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion," *Waste Manag,* vol. 50, pp. 264-74, Apr 2016.

[2] A. Degueurce, S. Picard, P. Peu, and A. Trémier, "Storage of Food Waste: Variations of Physical–Chemical Characteristics and Consequences on Biomethane Potential," *Waste and Biomass Valorization,* vol. 11, no. 6, pp. 2441-2454, 2019.

[3] F. Lu, X. Xu, L. Shao, and P. He, "Importance of storage time in mesophilic anaerobic digestion of food waste," *J Environ Sci (China),* vol. 45, pp. 76-83, Jul 2016.

[4] H. Fisgativa, A. Tremier, S. Le Roux, C. Bureau, and P. Dabert, "Understanding the anaerobic biodegradability of food waste: Relationship between the typological, biochemical and microbial characteristics," *J Environ Manage,* vol. 188, pp. 95-107, Mar 1 2017.

[5] R. Zhang *et al.*, "Characterization of food waste as feedstock for anaerobic digestion," *Bioresour Technol,* vol. 98, no. 4, pp. 929-35, Mar 2007.

[6] S. Heaven, Y. Zhang, R. Arnold, T. Paavola, F. Vaz, and C. Cavinato, "Valorgas Seventh Framework Programme Theme Energy: Composition analysis of food waste from study sites in geographically distinct regions of Europe," 2011.

[7] Y. Zhang, S. Kusch-Brandt, S. Heaven, and C. J. Banks, "Effect of Pasteurisation on Methane Yield from Food Waste and Other Substrates in Anaerobic Digestion," *Processes,* vol. 8, no. 11, 2020.

[8] H. Song, Y. Zhang, S. Kusch-Brandt, and C. J. Banks, "Comparison of Variable and Constant Loading for Mesophilic Food Waste Digestion in a Long-Term Experiment," *Energies,* vol. 13, no. 5, 2020.

[9] G. Srisowmeya, M. Chakravarthy, and G. Nandhini Devi, "Critical considerations in two-stage anaerobic digestion of food waste – A review," *Renewable and Sustainable Energy Reviews,* vol. 119, 2020.

[10] W. S. Lee, A. S. M. Chua, H. K. Yeoh, and G. C. Ngoh, "A review of the production and applications of waste-derived volatile fatty acids," *Chemical Engineering Journal,* vol. 235, pp. 83-99, 2014.

[11] S. Bengtsson, J. Hallquist, A. Werker, and T. Welander, "Acidogenic fermentation of industrial wastewaters: Effects of chemostat retention time and pH on volatile fatty acids production," *Biochemical Engineering Journal,* vol. 40, no. 3, pp. 492-499, 2008.

[12] M. Coma *et al.*, "Organic waste as a sustainable feedstock for platform chemicals," *Faraday Discuss,* vol. 202, pp. 175-195, Sep 21 2017.

[13] E. Jankowska, J. Chwialkowska, M. Stodolny, and P. Oleskowicz-Popiel, "Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH," *Chemical Engineering Journal,* vol. 326, pp. 901-910, 2017.

[14] E. Jankowska, A. Duber, J. Chwialkowska, M. Stodolny, and P. Oleskowicz-Popiel, "Conversion of organic waste into volatile fatty acids – The influence of process operating parameters," *Chemical Engineering Journal,* vol. 345, pp. 395-403, 2018.

[15] A. Perimenis, T. Nicolay, M. Leclercq, and P. A. Gerin, "Comparison of the acidogenic and methanogenic potential of agroindustrial residues," *Waste Manag,* vol. 72, pp. 178-185, Feb 2018.

[16] D. Shen *et al.*, "Acidogenic fermentation characteristics of different types of protein-rich substrates in food waste to produce volatile fatty acids," *Bioresour Technol,* vol. 227, pp. 125-132, Mar 2017.

[17] F. C. Silva, L. S. Serafim, H. Nadais, L. Arroja, and I. Capelaa, "Acidogenic fermentation towards valorisation of organic waste streams into volatile fatty acids," *Chem. Biochem. Eng. Q.,* vol. 27, no. 4, pp. 467-476, 2013.

[18] S. E. Daly, J. G. Usack, L. A. Harroff, J. G. Booth, M. P. Keleman, and L. T. Angenent, "Systematic Analysis of Factors That Affect Food-Waste Storage: Toward Maximizing Lactate Accumulation for Resource Recovery," *ACS Sustainable Chemistry & Engineering,* vol. 8, no. 37, pp. 13934-13944, 2020.
[19] L. Chatellard, E. Trably, and H. Carrere, "The type of carbohydrates specifically selects microbial community structures and fermentation patterns," *Bioresour Technol,* vol. 221, pp. 541-549, Dec 2016.
[20] A. Duber, R. Zagrodnik, J. Chwialkowska, W. Juzwa, and P. Oleskowicz-Popiel, "Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation," *Sci Total Environ,* vol. 728, p. 138814, Aug 1 2020.
[21] I. Angelidaki *et al.*, "Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays," *Water Sci Technol,* vol. 59, no. 5, pp. 927-34, 2009.
[22] F. Raposo *et al.*, "Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study," *Journal of Chemical Technology & Biotechnology,* vol. 86, no. 8, pp. 1088-1098, 2011.
[23] V. De Groof, M. Coma, T. C. Arnot, D. J. Leak, and A. B. Lanham, "Adjusting Organic Load as a Strategy to Direct Single-Stage Food Waste Fermentation from Anaerobic Digestion to Chain Elongation," *Processes,* vol. 8, no. 11, 2020.
[24] V. De Groof, M. Coma Bech, T. Arnot, D. Leak, and A. Lanham, "Selecting fermentation products for food waste valorisation with HRT and OLR as the key operational parameters [submitted]," *Waste Management,* 2020.
[25] R Core Team, "R: A language and envionment for statistical computing," ed. Vienna, Austria: R Foundation for Statistical Computing, 2017.
[26] F. E. Harrell Jr and C. Dupont, "Package 'Hmisc' version 4.4-1," ed: CRAN, 2020.
[27] T. Wei and V. Simko, "R package "corplot": Visualization of a Correlation Matrix (version 0.84)," ed, 2017.
[28] Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," *Journal of the Royal Statistical Society. Series B (Methodological),* vol. 57, no. 1, pp. 289-300, 1995.
[29] APHA, "Standard methods for the examination of water and wastewater," vol. 23, 2017.
[30] G. Manni and F. Caron, "Calibration and determination of volatile fatty acids in waste leachates by gas chromatography," *Journal of Chromatography A,* vol. 690, pp. 237-242, 1995.
[31] M. Albertsen, S. M. Karst, A. S. Ziegler, R. H. Kirkegaard, and P. H. Nielsen, "Back to Basics-The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities," *PLoS One,* vol. 10, no. 7, p. e0132783, 2015.
[32] R. Lucas, J. Groeneveld, H. Harms, K. Johst, K. Frank, and S. Kleinsteuber, "A critical evaluation of ecological indices for the comparative analysis of microbial communities based on molecular datasets," *FEMS Microbiol Ecol,* vol. 93, no. 1, Jan 2017.
[33] S. H. Hurlbert, "The nonconcept of species diversity: a critique and alternative parameters," *Ecology,* vol. 52, pp. 577-586, 1971.
[34] L. Jost, "Entropy and diversity," *Oikos,* vol. 113, no. 2, pp. 363-375, 2006.
[35] C. O. Nzeteu, A. C. Trego, F. Abram, and V. O'Flaherty, "Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system," *Biotechnol Biofuels,* vol. 11, p. 108, 2018.
[36] S. Dahiya, O. Sarkar, Y. V. Swamy, and S. Venkata Mohan, "Acidogenic fermentation of food waste for volatile fatty acid production with co-generation of biohydrogen," *Bioresour Technol,* vol. 182, pp. 103-113, Apr 2015.

[37] E. den Boer *et al.*, "Volatile fatty acids as an added value from biowaste," *Waste Manag,* vol. 58, pp. 62-69, Dec 2016.
[38] C. A. Contreras-Davila, V. J. Carrion, V. R. Vonk, C. N. J. Buisman, and D. Strik, "Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation," *Water Res,* vol. 169, p. 115215, Feb 1 2020.
[39] S. J. Lim, B. J. Kim, C. M. Jeong, J. D. Choi, Y. H. Ahn, and H. N. Chang, "Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor," *Bioresour Technol,* vol. 99, no. 16, pp. 7866-74, Nov 2008.
[40] H. Yesil, A. E. Tugtas, A. Bayrakdar, and B. Calli, "Anaerobic fermentation of organic solid wastes: volatile fatty acid production and separation," *Water Sci Technol,* vol. 69, no. 10, pp. 2132-8, 2014.
[41] T. I. M. Grootscholten, F. Kinsky dal Borgo, H. V. M. Hamelers, and C. J. N. Buisman, "Promoting chain elongation in mixed culture acidification reactors by addition of ethanol," *Biomass and Bioenergy,* vol. 48, pp. 10-16, 2013.
[42] X. Gómez, M. J. Cuetos, J. I. Prieto, and A. Morán, "Bio-hydrogen production from waste fermentation: Mixing and static conditions," *Renewable Energy,* vol. 34, no. 4, pp. 970-975, 2009.
[43] I. O. Bolaji and D. Dionisi, "Acidogenic fermentation of vegetable and salad waste for chemicals production: Effect of pH buffer and retention time," *Journal of Environmental Chemical Engineering,* vol. 5, no. 6, pp. 5933-5943, 2017.
[44] A. Duber *et al.*, "Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey," *Green Chemistry,* vol. 20, no. 16, pp. 3790-3803, 2018.
[45] V. Cabbai, M. Ballico, E. Aneggi, and D. Goi, "BMP tests of source selected OFMSW to evaluate anaerobic codigestion with sewage sludge," *Waste Manag,* vol. 33, no. 7, pp. 1626-32, Jul 2013.
[46] J. H. Ebner, R. A. Labatut, J. S. Lodge, A. A. Williamson, and T. A. Trabold, "Anaerobic co-digestion of commercial food waste and dairy manure: Characterizing biochemical parameters and synergistic effects," *Waste Manag,* vol. 52, pp. 286-94, Jun 2016.
[47] J. Tang, X. Wang, Y. Hu, Y. Zhang, and Y. Li, "Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR," *Waste Manag,* vol. 52, pp. 278-85, Jun 2016.
[48] M. T. Agler, B. A. Wrenn, S. H. Zinder, and L. T. Angenent, "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform," *Trends Biotechnol,* vol. 29, no. 2, pp. 70-8, Feb 2011.
[49] S. Im, M.-K. Lee, Y.-M. Yun, S.-K. Cho, and D.-H. Kim, "Effect of storage time and temperature on hydrogen fermentation of food waste," *International Journal of Hydrogen Energy,* vol. 45, no. 6, pp. 3769-3775, 2020.
[50] F. Asunis *et al.*, "Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey," *Bioresour Technol,* vol. 289, p. 121722, Oct 2019.
[51] N. Azbar, P. Ursillo, and R. E. Speece, "Effect of process configuration and substrate complexity on the performance of anaerobic processes," *Water Research,* vol. 35, no. 3, pp. 817-829, 2001.
[52] J. Dolfing, "The Microbial Logic behind the Prevalence of Incomplete Oxidation of Organic Compounds by Acetogenic Bacteria in Methanogenic Environments," *Microb Ecol,* vol. 41, no. 2, pp. 83-89, Feb 2001.
[53] D. G. Cirne, A. Lehtomaki, L. Bjornsson, and L. L. Blackall, "Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops," *J Appl Microbiol,* vol. 103, no. 3, pp. 516-27, Sep 2007.
[54] M. Zhou, B. Yan, J. W. C. Wong, and Y. Zhang, "Enhanced volatile fatty acids production from anaerobic fermentation of food waste: A mini-review focusing on

acidogenic metabolic pathways," *Bioresour Technol,* vol. 248, no. Pt A, pp. 68-78, Jan 2018.
[55] M. G. Ganzle and R. Follador, "Metabolism of oligosaccharides and starch in lactobacilli: a review," *Front Microbiol,* vol. 3, p. 340, 2012.
[56] H. Wang *et al.*, "Improvement of n-caproic acid production with Ruminococcaceae bacterium CPB6: selection of electron acceptors and carbon sources and optimization of the culture medium," *Microb Cell Fact,* vol. 17, no. 1, p. 99, Jun 25 2018.
[57] S. Esquivel-Elizondo *et al.*, "The isolate Caproiciproducens sp. 7D4C2 produces n-caproate at mildly acidic conditions from hexoses: genome and rBOX comparison with related strains and chain-elongating bacteria," *Biotechnology for Biofuels,* 2020.
[58] R. Nelson, D. Peterson, E. Karp, G. Beckham, and D. Salvachúa, "Mixed Carboxylic Acid Production by Megasphaera elsdenii from Glucose and Lignocellulosic Hydrolysate," *Fermentation,* vol. 3, no. 1, 2017.
[59] J. L. Rombouts, E. M. M. Kranendonk, A. Regueira, D. G. Weissbrodt, R. Kleerebezem, and M. C. M. van Loosdrecht, "Selecting for lactic acid producing and utilising bacteria in anaerobic enrichment cultures," *Biotechnol Bioeng,* vol. 117, no. 5, pp. 1281-1293, May 2020.
[60] P. Candry, L. Radic, J. Favere, J. M. Carvajal-Arroyo, K. Rabaey, and R. Ganigue, "Mildly acidic pH selects for chain elongation to caproic acid over alternative pathways during lactic acid fermentation," *Water Res,* vol. 186, p. 116396, Sep 7 2020.
[61] B. Liu, S. Kleinsteuber, F. Centler, H. Harms, and H. Strauber, "Competition Between Butyrate Fermenters and Chain-Elongating Bacteria Limits the Efficiency of Medium-Chain Carboxylate Production," *Front Microbiol,* vol. 11, p. 336, 2020.
[62] A. Detman, D. Mielecki, A. Chojnacka, A. Salamon, M. K. Blaszczyk, and A. Sikora, "Cell factories converting lactate and acetate to butyrate: Clostridium butyricum and microbial communities from dark fermentation bioreactors," *Microb Cell Fact,* vol. 18, no. 1, p. 36, Feb 13 2019.
[63] B. E. Jackson and M. J. McInerney, "Anaerobic microbial metabolism can proceed close to thermodynamic limits," *Nature,* vol. 415, pp. 454-456, 2002.
[64] C. M. Spirito, H. Richter, K. Rabaey, A. J. Stams, and L. T. Angenent, "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr Opin Biotechnol,* vol. 27, pp. 115-22, Jun 2014.
[65] Q. Wu *et al.*, "Concentrating lactate-carbon flow on medium chain carboxylic acids production by hydrogen supply," *Bioresour Technol,* vol. 291, p. 121573, Nov 2019.
[66] T. Noike and O. Mizuno, "Hydrogen fermentation of organic municipal wastes," *Water Science and Technology,* vol. 42, no. 11, pp. 155-162, 2000.
[67] N. H. Yasin, T. Mumtaz, M. A. Hassan, and N. Abd Rahman, "Food waste and food processing waste for biohydrogen production: a review," *J Environ Manage,* vol. 130, pp. 375-85, Nov 30 2013.
[68] K. Feng, H. Li, and C. Zheng, "Shifting product spectrum by pH adjustment during long-term continuous anaerobic fermentation of food waste," *Bioresour Technol,* vol. 270, pp. 180-188, Dec 2018.
[69] Y. Wu, H. Ma, M. Zheng, and K. Wang, "Lactic acid production from acidogenic fermentation of fruit and vegetable wastes," *Bioresour Technol,* vol. 191, pp. 53-8, Sep 2015.
[70] E. Santillan, H. Seshan, F. Constancias, and S. Wuertz, "Trait-based life-history strategies explain succession scenario for complex bacterial communities under varying disturbance," *Environ Microbiol,* vol. 21, no. 10, pp. 3751-3764, Oct 2019.
[71] J. De Vrieze *et al.*, "Stochasticity in microbiology: managing unpredictability to reach the Sustainable Development Goals," *Microb Biotechnol,* vol. 13, no. 4, pp. 829-843, Jul 2020.

[72] D. J. Anneken, S. Both, R. Christoph, G. Fieg, U. Steinberner, and A. Westfechtel, "Fatty Acids," in *Ullmann's Encyclopedia of Industrial Chemistry*, vol. 14Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 2006, pp. 73-116.
[73] The Merck Index, "The Merck Index online - An Encyclopedia of Chemicals, Drugs, and Biologicals," ed. Whitehouse Station: Merck Sharp & Dohme Corp., 2013.
[74] C. S. Lopez-Garzon and A. J. Straathof, "Recovery of carboxylic acids produced by fermentation," *Biotechnol Adv,* vol. 32, no. 5, pp. 873-904, Sep-Oct 2014.
[75] K. Choi, B. S. Jeon, B. C. Kim, M. K. Oh, Y. Um, and B. I. Sang, "In situ biphasic extractive fermentation for hexanoic acid production from sucrose by Megasphaera elsdenii NCIMB 702410," *Appl Biochem Biotechnol,* vol. 171, no. 5, pp. 1094-107, Nov 2013.
[76] K. L. Wasewar, D. Shende, and A. Keshav, "Reactive extraction of itaconic acid using tri-n-butyl phosphate and aliquat 336 in sunflower oil as a non-toxic diluent," *Journal of Chemical Technology & Biotechnology,* vol. 86, no. 2, pp. 319-323, 2011.
[77] B. M. Dolman, C. Kaisermann, P. J. Martin, and J. B. Winterburn, "Integrated sophorolipid production and gravity separation," *Process Biochemistry,* vol. 54, pp. 162-171, 2017.
[78] J. Xu, J. J. Guzman, S. J. Andersen, K. Rabaey, and L. T. Angenent, "In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis," *Chem Commun (Camb),* vol. 51, no. 31, pp. 6847-50, Apr 21 2015.
[79] Y. Liu, P. He, L. Shao, H. Zhang, and F. Lu, "Significant enhancement by biochar of caproate production via chain elongation," *Water Res,* vol. 119, pp. 150-159, Aug 1 2017.

Chapter 7. Conclusions and future work

This chapter contains a concluding overview of the main findings of this thesis and their contributions to the field of bio-waste valorisation by mixed anaerobic processes. The initial thesis goals and objectives are assessed and a future perspective is provided on how the research could be carried forward to advance in the field of anaerobic microbial processes for bio-waste valorisation.

This thesis explored the potential of mixed culture fermentation for bio-waste valorisation. In particular, it focussed on the production of medium chain carboxylic acids (MCCA) from mixed food waste (FW) fermentation in a single-stage stirred tank reactor (STR). MCCA were targeted due to their potential for easier separation and higher economic value compared to other acidogenic fermentation (AF) products. Processes producing MCCA are typically complex. The need to invest in specific infrastructure and develop new expertise hinder the implementation of novel technologies in the waste management sector. Therefore, a reactor setup was used that was similar to those established in current anaerobic digestion (AD) treatment facilities. The feedstock (i.e., FW), relevant for the industry, was facilitated by the partnership with Wessex Water and GENeco (Avonmouth, UK).

Three general goals were set to evaluate the production of MCCA from mixed culture fermentation of FW. The first goal was to acquire knowledge on chain elongation in microbial communities fed with complex organic feedstock. This was addressed in the literature study (Chapter 2) that characterized current research gaps. The second goal was identifying operating strategies that enable microbial chain elongation in FW fermentation with minimal chemical addition in a single-stage STR. This was addressed by outlining four specific research objectives explored in the next four research chapters (Chapters 3-6). The findings from addressing these four objectives resulted in outlining an operating guideline for production of MCCA from food waste, presented in the following section. The third goal, investigation of the MMC and underlying fermentation pathways, was defined in two more research objectives (5 and 6, respectively). These were evaluated in parallel with the other objectives in the same research chapters by including microbial community analysis, cycle studies and batch studies.

7.1. Towards an operating guideline to target MCCA in single-stage acidogenic food waste fermentation

The combination of the operating strategies found from the literature review and the experimental work from this thesis can be summarised into a set of practical guidelines. These target MCCA in mixed culture fermentation of FW, in line with the second goal of this thesis (Figure 7-1).

The literature study (Chapter 2) concluded that operating at a pH of 5.5, mesophilic temperature around 35°C and an organic overload at start-up (≥ 5 $gCOD_{fed}$ per $gVS_{inoculum}^{-1}$) were suitable to switch from methane to MCCA production. In Chapter 3, these operating parameters were tested and the first research objective addressed, i.e., exploring an

organic overload as strategy to steer an AD community in long-term operation of a single-stage STR towards chain elongation with minimal chemical addition. The start-up strategy of higher F/M (8.4 gCOD gVS^{-1}) and operating at higher OLR (depending on feedstock from 8.5 up to 21 ± 2 gCOD L^{-1} d^{-1}), and indirectly lower HRT (18 to 14 ± 2 d) compared to the AD reactor operated in parallel (0.8 gCOD gVS^{-1}, 4.2 to 4.4 gCOD L^{-1} d^{-1} and 35 to 69 ± 6 d), led to a net production of MCCA and inhibited methanogenesis. The high organic load allowed producing MCCA at concentrations similar to more complicated reactor setups (22 ± 4 gCOD L^{-1} for n-caproic and 7 ± 2 gCOD L^{-1} for n-caprylic acid). However, it also resulted to reduced degradation of the FW solids in comparison with AD processes. Thus, for waste management purposes, hydrolysis and yields of single-stage MCCA production need to improve. Alternatively, this process could fit within a broader biorefinery context that includes post chain elongation treatment by, e.g., AD processes that stabilise the solids and produce biogas from the remaining organics.

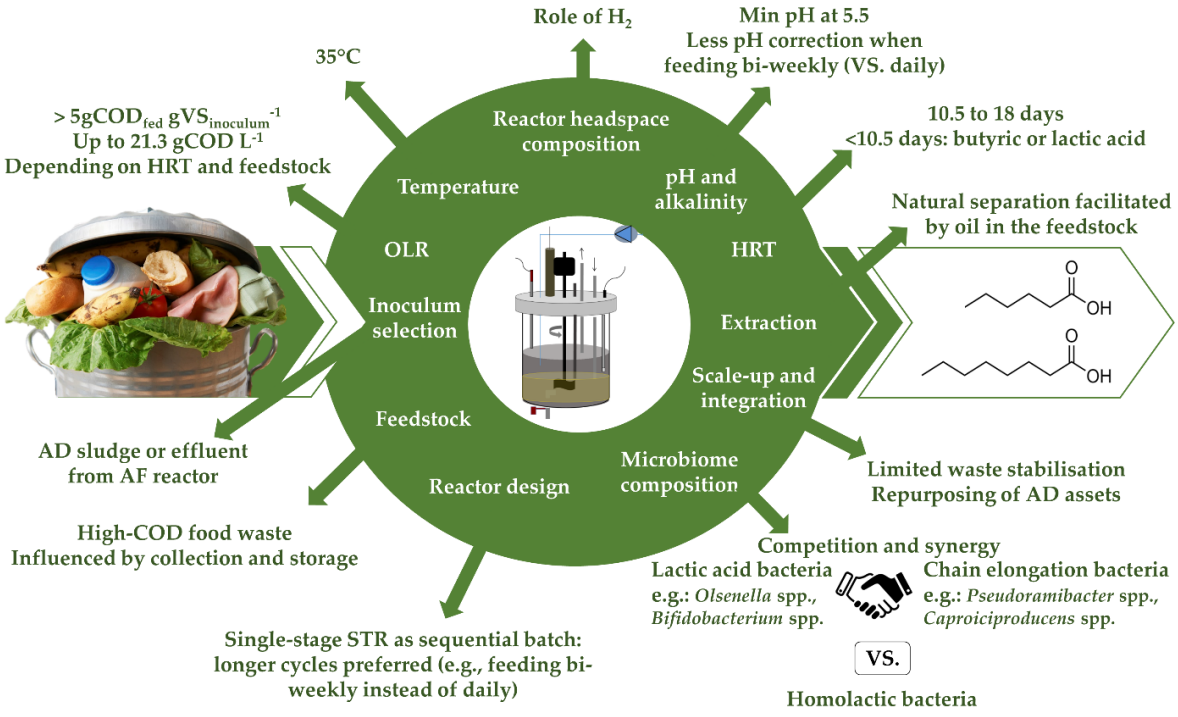


Figure 7-1 *Simplified overview of parameters considered in this thesis for the production of MCCA from acidogenic food waste fermentation.*

Chapter 4 addressed the second research objective, evaluating the effect of HRT and OLR on fermentation. It was found that operating at an OLR of 12 gCOD $L^{-1}d^{-1}$ and HRT of 8.5 days gave n-butyric acid as the main carboxylic acid. At the same HRT of 8.5 days and operating at an OLR of 20 gCOD $L^{-1}d^{-1}$, similar to the OLR that stimulated chain elongation in Chapter 3, gave predominantly lactic acid. Chain elongation was stimulated instead, when reactors were operated at a higher HRT of 10.5 days keeping OLR at 12 gCOD $L^{-1}d^{-}$

[1] (n-caproic acid up to 13.6 gCOD L^{-1}). Microbial community analysis revealed that at shorter HRT, the relative abundance of genera related to chain elongation and other types of secondary fermentation were much lower, whereas homolactic fermentation dominated at higher OLR. Thus, while in Chapter 3 organic overload stimulated chain elongation, Chapter 4 uncovered that a high OLR should not come at the cost of lowering HRT. Understanding the interplay between HRT and OLR on MCCA production is crucial for industrial operation as they are the parameters most commonly used to exert control on industrial-scale AD processes. The three different types of fermentation that were achieved by operating at different combination of HRT and OLR highlighted the potential for a flexible product portfolio, and thus the attractiveness of acidogenic fermentation for bio-waste valorisation.

Chapter 5 tackled the third research objective, evaluating the effect of a semi-continuous feeding pattern. The discussion from the literature review (Chapter 2) hypothesised this could stimulate chain elongation in a single-stage system. Feeding twice a week was compared with feeding daily in semi-continuous stirred tank reactor while operating at an HRT and OLR suitable for chain elongation (10.5 days, 9 to 13 ± 2 gCOD $L^{-1}d^{-1}$). Operating with bi-weekly feeding gave a more stable product profile and reduced the need for pH-correcting chemicals compared to daily feeding. A more fluctuating lactic acid content was observed in the effluent of the daily-fed reactors since the greater availability of easily biodegradable sugars favoured homolactic fermentation over heterolactic acetate-ethanol fermentation with chain elongation. Thus, semi-continuous operation is more favourable for MCCA production from FW than continuous feeding.

In Chapter 6, the composition of the FW feedstock was evaluated for variation and its impact on fermentation, thereby addressing the fourth research objective. This chapter revealed that the source of the feedstock affects acidogenic fermentation. For instance, fresh cafeteria FW will more easily accumulate lactic acid whereas pre-processed FW as obtained from recycling centres will naturally accumulate carboxylic acids. If aiming at MCCA production, fresh and highly biodegradable FW might require a two-step process (with pre-fermentation) or lower OLR and/or longer HRT compared to FW that has undergone some degradation during storage. Alternatively, it provides interesting research routes of fine-tuning the feedstock depending on which fermentation product is targeted.

7.2. Community analysis and cycle studies elucidate the microbial pathways

A clear outcome from the literature review (Chapter 2) is a need to better understand the underlying mechanisms to produce MCCA in MMC fermentation of complex bio-waste,

which set the base for the third thesis goal. It was addressed throughout the different chapters by analysing the composition of the MMC, as specified by the fifth research objective, and studying the succession of conversion reactions in cycle and batch studies, in line with the sixth research objective.

From the literature review, it was postulated that competing reactions with chain elongation would be methanogenesis towards methane and lactate reduction to produce propionic acid. Indeed, methanogenesis persisted in some experiments even when applying an organic overload, specifically hydrogenotrophic methanogenesis (Chapter 3). However, limited propionic acid was produced. This selected for even-chained MCCA, even for reactor systems where lactic acid accumulated (Chapter 5). Contrarily, we observed an additional competition between chain elongation and lactic acid accumulation. Namely, fermenting fresh cafeteria FW with soybean wastewater (Chapter 5) or mixed FW with additional sucrose (Chapters 6) gave an alternating fermentation outcome of either lactic acid accumulation or chain elongation. This was reflected by changes in the microbial community. On one side, homolactic *Lactobacillus* spp. performed acidogenic lactic acid fermentation hindering other types of fermentation. On the other side, a partnership between other lactic acid bacteria (LAB), such as *Olsenella* spp. and *Bifidobacterium* spp., with chain elongation bacteria, e.g., *Pseudoramibacter* spp. and *Caproiciproducens* spp., allowed to accumulate MCCA. This was attributed to homolactic LAB having a kinetic advantage over other fermentative bacteria in complex, sugar-rich media. By operating at lower OLR (Chapter 4) or by feeding semi-continuously with longer feeding cycles, e.g., bi-weekly feeding compared to daily (Chapter 5), the competitive advantage of homolactic LAB could be countered. By operating at longer HRT, secondary fermentation such as chain elongation bacteria were stimulated (Chapter 4), i.e., a sequence of 1) hydrolysis, 2) primary fermentation to accumulate VFA and electron donors to 3) chain elongation. Thus, this thesis exposed some of the competitive and synergistic pathways occurring in acidogenic FW fermentation in a single-stage reactor, and how understanding them allows to adjust operating strategy.

7.3. The opportunities for high-COD and oily food waste

The reactor experiment discussed in Chapter 3 indicated the potential of FW with a high COD (297 ± 9 gCOD L^{-1}) as a preferred feedstock for MCCA production in single-stage STR since it allowed a high organic load and long HRT. Contrarily, in the AD reactor also operated in Chapter 3, switching to this high-COD FW crashed methane production due to an organic overload for methanogens. By addressing the fourth research objective in Chapter 6, it was found that this FW with high COD was an outlier in terms of COD content

compared to what is generally available in the targeted AD plant for FW recycling (average of collections at 163 ± 55 gCOD L^{-1}). This particular FW collection was high in COD likely due to mixtures containing oily washings from food processing. By contrast, addition of sucrose to increase COD and biodegradability of the FW did not enhance chain elongation and instead resulted in process instability due to lactic acid accumulation by providing homolactic bacteria with a competitive advantage.

Aside from enhancing elongation by supplementing organics, it was discussed in Chapter 6 that the presence of oils resulted into separation from the bulk fermentation broth and extraction of MCCA into an immiscible layer due to their hydrophobicity. This is an interesting finding as it presents two new opportunities for oily FW, which is generally a difficult waste to treat. Firstly, one could work towards defining an optimal FW mixture to exert a high OLR with sufficiently long retention times to inhibit methanogenesis and stimulate chain elongation instead of lactic acid. Secondly, it could provide an *in situ* separating mechanism, potentially lowering toxicity effects and facilitating downstream processing. Further research is required to conclude the initial findings of this thesis.

7.4. Perspective: A highly interdisciplinary route towards a new bio-waste valorisation technology

The development of predictable and stable MMC fermentation processes where the target product can be adjusted according to market demand will require improved monitoring of the feedstock composition, understanding the microbial ecology, and expansion of downstream processing to generate marketable products.

Optimising composition of the feedstock (e.g., mixing waste streams) or developing specific downstream processes will only be worthwhile if the value gained from the fermentation products outweighs the increased process complexity. A system-based approach will be required where bio-waste is regarded as a resource with its own supply chain. Thus, waste treatment becomes an analogue to a bio-refinery approach where value is maximised. Careful monitoring of these processes by, for instance, assessing their life cycle, will be critical to ensure the most sustainable and circular value chains are formed. For instance, the environmental cost of transporting bio-waste or the use of arable land and water to grow crops for direct use as feedstock instead for food production are some potential pitfalls.

As the work in this thesis showed, optimising process design requires understanding how the changes in operating conditions steer the MMC and resulting fermentation outcome. The amount of “omics” approaches is rapidly evolving thereby improving the robustness of analysis and the level of possible interpretation. Yet, with this comes increased complexity

of the data generated. A collaborative approach is required between method improvement for community monitoring, advanced statistical data processing and programming, microbial ecology and reactor engineering. Luckily, as these techniques are maturing, more and more information is made accessible. For instance, various of the statistics and programming resources used in this thesis for microbial community analysis was possible thanks to researchers sharing software and writing blogs on platforms such as GitHub. Increased data sharing is allowing to pool results from different studies together and reach overarching conclusions. For instance, the MiDAS (Microbial Database for Activated Sludge) field guide is such a project aiming to collate the knowledge of the microbial communities present in wastewater treatment. It was in the light of open data sharing that all data from published material of this thesis was made publically available on the University of Bath data archive or on the European Nucleotide Archive for sequenced data. Increased data sharing and collaboration will be key to deepen the collective understanding of microbial ecology and how it can be applied.

Lastly, product separation and formulation to generate a marketable product range will need to be developed before this technology can be implemented. While various publications state a wide range of applications for MCCA, sourcing them from mixed bio-waste will inherently limit certain applications. For instance, high purities could be required for chemical synthesis or, health and safety concerns will exclude the use of waste-derived caprylic acid as food supplement. Additional product development and market research will be key in unlocking how anaerobic mixed culture processes can fit within a global scope and be a sustainable bio-waste valorisation technology.